Environmental Microbiology

A Laboratory Manual

SECOND EDITION: 2004

Environmental Microbiology

A Laboratory Manual

SECOND EDITION: 2004

I.L. Pepper and C.P. Gerba

Photography and Technical Editor: K.L. Josephson
Copy Editor: E.R. Loya

ELSEVIER
ACADEMIC
PRESS

AMSTERDAM • BOSTON • HEIDELBERG • LONDON
NEW YORK • OXFORD • PARIS • SAN DIEGO
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO

Acquisition Editor: David Cella
Project Manager: Sarah Hajduk
Assistant Editor: Kelly Sonnack
Marketing Manager: Linda Beattie
Cover Design: Linda Rettich
Interior Design: Accurate Art, Inc.
Composition: SNP Best-Set
Printer: The Maple-Vail Book Manufacturing Group
Cover Printer: Phoenix Color

Elsevier Academic Press
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

This book is printed on acid-free paper. ∞

Library of Congress Cataloging-in-Publication Data
APPLICATION SUBMITTED

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN: 0-12-550656-2

For all information on all Elsevier Academic Press publications
visit our Web site at www.books.elsevier.com

Printed in the United States of America
04 05 06 07 08 09 9 8 7 6 5 4 3 2 1

For my bravest dog—Moss. Her tag read: “Be kind—I’m blind.” You know—you can learn an awful lot from a blind dog that loves you.

Ian Pepper, August 3, 2004

To Peggy, Peter and Phillip.

Chuck Gerba, August 3, 2004

Table of Contents

SECTION FIVE

Preface

BASICS

This manual has been designed for upper division and/or graduate-level laboratory sessions in environmental microbiology. Overall, this Environmental Microbiology Laboratory Manual is optimally designed for use with students that are concurrently taking a lecture class in Environmental Microbiology, using the text "Environmental Microbiology" (R.M. Maier, I.L. Pepper, and C.P. Gerba—Academic Press).

Section One • Basic Protocols

These first two experiments introduce students to two concepts that are critical to many of the subsequent experiments outlined in this manual.

Experiment 1 introduces students to the basic concepts of bacterial growth in pure culture. These concepts are illustrated using standard broth culture and dilution and plating techniques. Experiment 2 demonstrates how to measure soil moisture content, and discusses the significance of soil moisture on soil microbial activity.

Section Two • Examination of Soil Microorganisms Via Microscopic and Cultural Assays

Experiments 3–6 are related to analysis and study of microorganisms in soil. Experiment 3 introduces the student to soil as a habitat for microorganisms, the main types of soil microorganisms, and interactions between organisms and soil. Experiments 4–6 cover the main cultural enumeration techniques for soil microorganisms while introducing soil fungi, bacteria, actinomycetes, and algae in more detail.

Section Three • Microbial Transformations and Response to Contaminants

This section illustrates the microbial activity of bacteria in soil and water. Such activities not only affect nutrient cycling, but also interactions with organic and metal contaminants. Experiment 7 demonstrates the conversion of reduced forms of sulfur to sulfate, while Experiment 8 illustrates a method to monitor general metabolic activity via dehydrogenase activity. Experiment 9 documents the important autotrophic activities of nitrification, and subsequent denitrification which can be autotrophic or heterotrophic. Experiments 10, 11, and 12 illustrate bacterial responses to organic and metal contaminants. In contrast Experiments 13 and 14 evaluate uptake of assimilable carbon and oxygen.

Section Four • Water Microbiology

This section involves assays of microbial pathogens—bacteria, viruses, and protozoan parasites—used in water and food quality control. Experiments 15 and 16 teach basic methods for coliform detection and quantification in water. Experiment 17 illustrates the detection of bacteriophages. Contemporary methods for the rapid detection of coliforms are the subject of Experiments 18 and 19.

Section Five • Advanced Topics

These experiments require more sophisticated expertise and/or equipment. Experiments 20 and 21 outline procedures for the detection of enteric viruses and protozoan parasites. Experiment 22 looks at the topic of disinfection. In contrast, Experiment 23 illustrates procedures for the detection of airborne microorganisms. The final experiment involves molecular methods of detection and identification of bacteria.

Appendix 1 • Preparation of Media and Stains for Each Experiment

Appendix 2 • Glossary

A glossary is included that covers terms that may be new with this course as well as basic, discipline-specific terminology from microbiology and soil science that may be new to some students.

MANUAL CONVENTIONS

Each experiment generally contains the following sections:

Overview

A brief synopsis of the experiment designed to give the student the big picture.

Theory and Significance

This section describes biological, chemical, and physical principles behind the assays performed, how they relate to the environment, and the significance of the topic.

Procedure

The labs are broken up into multiple periods to facilitate the organization of experiments that can run concomitantly. A detailed description of the materials and equipment needed to carry out the experiment for each student is given at the head of each period.

An enumerated listing of each step involved in carrying out the experiment is enhanced with schematics summarizing the procedures involved in many experiments.

Tricks of the Trade

These are practical tips to help the student make the experiment successful. At first glance these seem very simplistic, but experience has shown the authors that these hints will prevent the mistakes that students have frequently made in the past, and would likely make again.

Potential Hazards

Safety aspects associated with the experiment are identified for the student.

Calculations

Calculations necessary for the analysis of experimentally determined data are assigned along with a discussion of the formulas used.

Questions and Problems

Assignments are available for the student to demonstrate an understanding of the material in each experiment.

References

A listing of useful articles and books is also supplied.

SUGGESTED SOIL TYPES AND TESTS

Soil Selection

Soils for the soil microbiology section should be chosen to represent as diverse a range of soil types as possible. Some suggestions for locating divergent soils include:

- Plowed agricultural land and adjacent, unplowed land.
- Mountain soil and valley soil.
- Arid soil and mountain top forest soil.
- Samples taken at distinct depths.

Experience has shown that coarse textured soils are easier to work with rather than fine textured clays. Soils are normally sieved (2 mm) and stored 4°C prior to use.

SECTION ONE

Basic Protocols



Dilution and Plating of Bacteria and Growth Curves

EXPERIMENT 1

1.1. OVERVIEW

Objective: ***To use dilution and plating of broth cultures of a bacterium to introduce students to cultural methodologies and concepts of bacterial growth.***

- Students will receive aliquots of broth cultures of *E. coli* that have been incubated for known but variable time intervals resulting in different concentrations of bacteria in broth.
- Aliquots are diluted and plated.
- After incubation and subsequent counting of the colonies on the plates, a growth curve is plotted and mean generation time calculated.

1.2. THEORY AND SIGNIFICANCE

Perhaps the most widely used technique for the study of bacteria is the growth of a microbe of interest in a liquid nutrient medium, followed by dilution and plating on a solid agar medium. Here the theory is that one colony arises from one organism. Each colony is then referred to as a **colony forming unit** (**CFU**). In addition to providing an estimate of bacterial numbers, this procedure allows the opportunity to obtain pure culture isolates. Oftentimes, researchers will measure the turbidity of the liquid culture at different time intervals using a spectrophotometer. The comparison of turbidity with plating results allows for a quick estimation of bacteria numbers in future studies. These techniques are used in all aspects of microbiology including clinical and environmental microbiology. Because of its importance this topic is introduced here as the first exercise in this laboratory manual. The growth of a bacterial isolate will be followed as a function of time to illustrate the various phases of growth that occur in liquid culture. Intuitively one can recognize that bacterial growth (via cell division) in liquid media will continue to occur until: a) nutrients become limiting; or b) microbial waste products accumulate and inhibit growth (Maier et al., 2000).

To understand and define the growth of a particular microorganism, cells are placed in a flask in which the nutrient supply and environmental conditions are controlled. If the liquid medium supplies all the nutrients required for growth and environmental parameters are conducive to growth, the increase in numbers can be measured as a function of time to obtain a growth curve. Several distinct growth phases can be observed within a growth curve (Figure 1-1). These include the lag phase, the exponential or log phase, the stationary phase, and the death phase. These phases correspond to distinct periods of growth and associated physiological changes (Table 1-1).

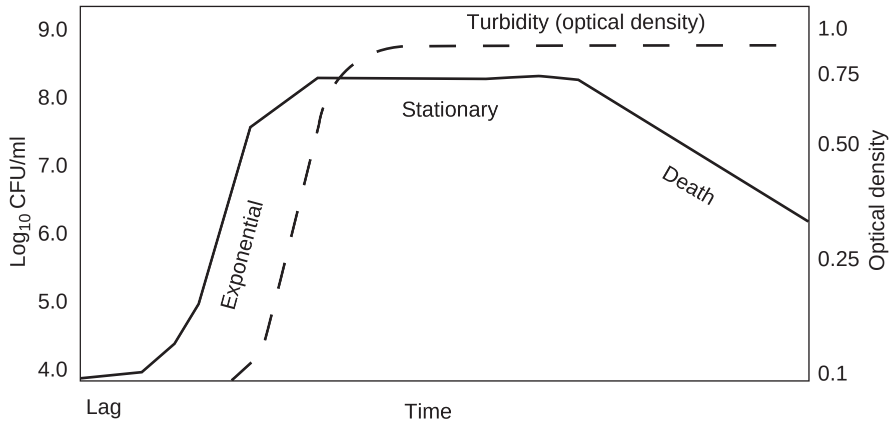


Figure 1-1 A typical growth curve for a bacterial population. Compare the difference in the shape of the curves in the death phase (colony-forming units (CFUs) versus optical density). The difference is due to the fact that dead cells still result in turbidity.

Table 1-1 The Four Phases of Bacterial Growth

Phase	Characteristics
1. Lag Phase	Slow growth or lack of growth due to physiological adaptation of cells to culture conditions or dilution of exoenzymes due to initial low cell densities.
2. Exponential or Log Phase	Optimal growth rates during which cell numbers double at discrete time intervals known as the mean generation time (Fig. 1-2).
3. Stationary Phase	Growth (cell division) and death of cells counterbalance each other resulting in no net increase in cell numbers.
4. Death Phase	Death rate exceeds growth rate resulting in a net loss of viable cells.

Theoretically, the time taken for cell division to occur is the **mean generation time** or **doubling time**. The mean generation time can be calculated through the use of a dilution and plating experiment. See Section 1.6 for an example calculation.

1.3. PROCEDURE

Pre Lab for Instructor Prior to Class

Materials

E. coli culture
trypticase soy broth[1]
50 ml flask
micropipettes

1 Difco Detroit, MI.

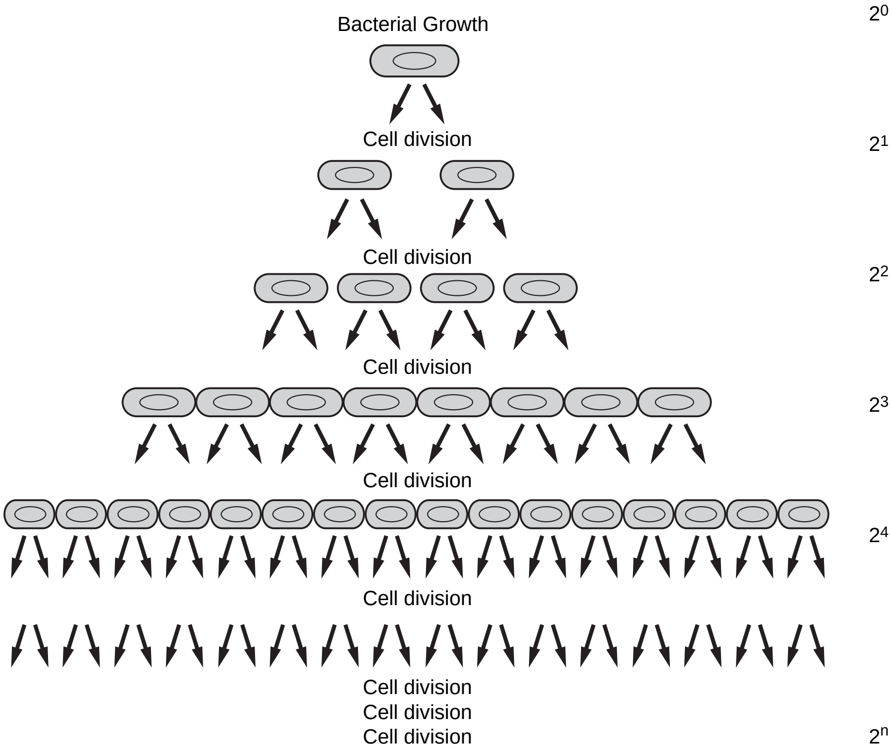


Figure 1-2 Exponential cell division. Each cell division results in a doubling of the cell number. At low cell numbers the increase is not very large, however after a few generations, cell numbers increase explosively. After n divisions we have 2^n cells.

2 days before experiment

Inoculate a 50 ml flask of trypticase soy broth (TSB) medium with *E. coli*. Incubate overnight at 27°C. This will yield 10^9 CFU/ml.

1 day before experiment

Use 100 µl of the prepared culture to inoculate 250 ml of TSB (in a 500 ml flask). Mix thoroughly and remove 5 ml and refrigerate immediately. This is T = 0 and will yield approximately 5×10^5 CFU/ml. Place the flask of *E. coli* in a 37°C shaking incubator. Remove 5 ml aliquots of culture every hour up to 8 hours. Store each aliquot at 4°C. These cultures should be designated T_0 through T_8.

First Period

Materials

1 ml aliquots of *E. coli* broth cultures (or other bacterium)
0.9 ml sterile water dilution tubes in microfuge tubes
micropipettes
poured Petri plates with trypticase soy agar
ice
glass hockey stick spreader
vortex mixer
gas burner
ethyl alcohol for flame sterilization

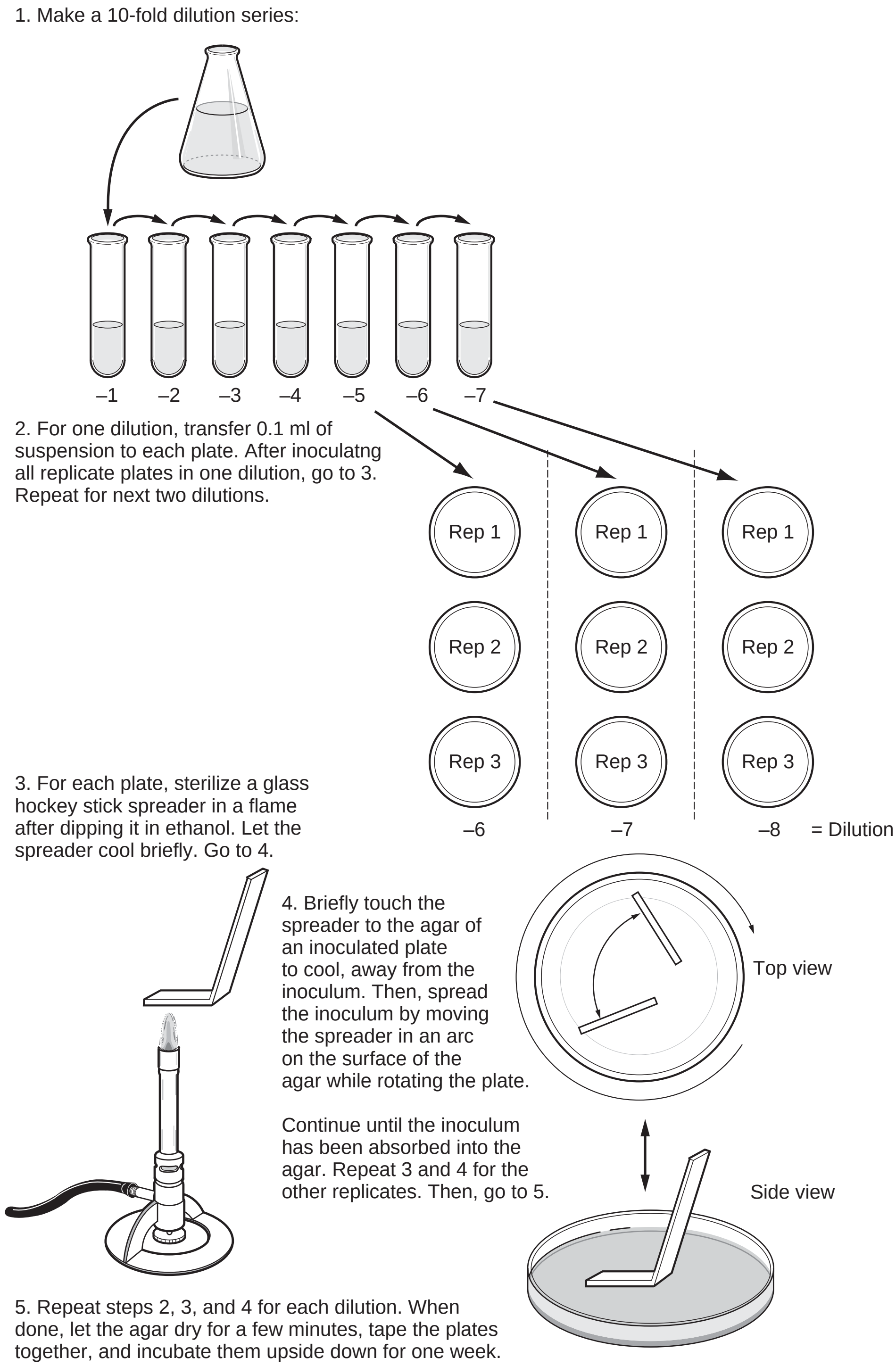


Figure 1-3 Schematic showing the procedure for counts of *E. coli*.

Remove aliquots of *E. coli* from the refrigerator and place on ice for transport to teaching lab. It may be desirable to split the 5 ml cultures into smaller volumes so each lab group has their own tube for assay. Keep all cultures on ice until use.

In Lab

Instructor: Each student can do all cultures (T_0 thru T_8) or different cultures can be assigned to different students (e.g., 2 cultures/student).

1. Set up a series of dilution tubes to obtain dilutions of 10^{-1} through 10^{-7} of the *E. coli* cultures. Microfuge tubes are convenient to do this (see Figure 1-3). Each dilution tube will have 900 µl of dilution fluid (sterile saline). A dilution series will be needed for each *E. coli* culture (T_0 thru T_8).

2. Begin dilutions by adding 100 µl of *E. coli* from the tube labeled T_0 which is the initial *E. coli* culture to tube A. Tube A is the 10^{-1} dilution of T_0.

3. Vortex the 10^{-1} tube for 5 seconds.

4. Follow this by subsequently adding 100 µl of Tube A to the next tube of saline (Tube B). Tube B is a 10^{-2} dilution of T_0. Repeat until completing the dilution series, referring to Table 1-2 to see how far you will need to make dilutions for each *E. coli* culture. Remember to vortex each tube prior to transfer. It is also important to use a new pipette tip for each transfer.

5. Repeat dilutions for T_1 through T_8 or for whatever samples were assigned to you. Again refer to Table 1-2 to see how far you need to make your dilutions.

6. Plate according to the regiment specified in Table 1-2.

7. Label plates with the dilution and volume to be added to the plate. Make sure the label contains the time point plated (T_1 thru T_8) identification. Use triplicate plates for each dilution.

8. Pipette 100 µl from each of the three dilutions to be plated. Add 100 µl of each dilution tube to be plated by pipetting the amount to the center of the agar plate (Figure 1-3).

9. Immediately spread the aliquot by utilizing a flame sterilized "L" shaped glass rod. If the aliquot is not spread immediately, it will sorb *in situ* in the plate resulting in bacterial overgrowth at the spot of initial inoculation.

10. Repeat the plating for each dilution series for T_1 through T_8 cultures. Remember to sterilize the rod in between plates and especially between different dilutions.

11. Once plates have dried for a few minutes, invert and place in 37°C incubator overnight. Following this, store plates in refrigerator until the next class period.

Table 1-2 Plating protocol for *E. coli* cultures

***E. coli* culture**	**Dilutions to be plated**		
T_0	10^{-1}	10^{-2}	10^{-3}
T_1	10^{-1}	10^{-2}	10^{-3}
T_2	10^{-2}	10^{-3}	10^{-4}
T_3	10^{-3}	10^{-4}	10^{-5}
T_4	10^{-4}	10^{-5}	10^{-6}
T_5	10^{-5}	10^{-6}	10^{-7}
T_6	10^{-6}	10^{-7}	10^{-8}
T_7*	10^{-5}	10^{-6}	10^{-7}
T_8*	10^{-4}	10^{-5}	10^{-6}

*Lower dilutions take into account lower populations due to death phase.

Second Period

1. Examine plates for uniformity of colonies and lack of contamination (see Figure 1-4).

2. For each culture (T_0 through T_8), count triplicate plates at one dilution that contains between 30 and 300 colonies.

3. Calculate the number of cells per ml of original culture for T_0 through T_8 cultures.

4. For example, the number of colonies resulting from a 10^{-4} dilution is 30, 28, and 32.

   Mean number of colonies = 30 colonies

   These arose from 0.1 ml of a 10^{-4} dilution

   $$\text{Number of colonies per ml} = \frac{30 \times 10^{-4}}{0.1}$$

5. Plot $\log_{10}$ CFU/ml versus time (hours).

6. From the graph, identify the exponential phase of growth. Using two time points within the exponential phase of growth and corresponding cell numbers, calculate the mean generation time.

1.4. TRICKS OF THE TRADE

DO:

- Keep broth cultures on ice until you dilute and plate
- Use multiple dilutions to ensure you get countable plates

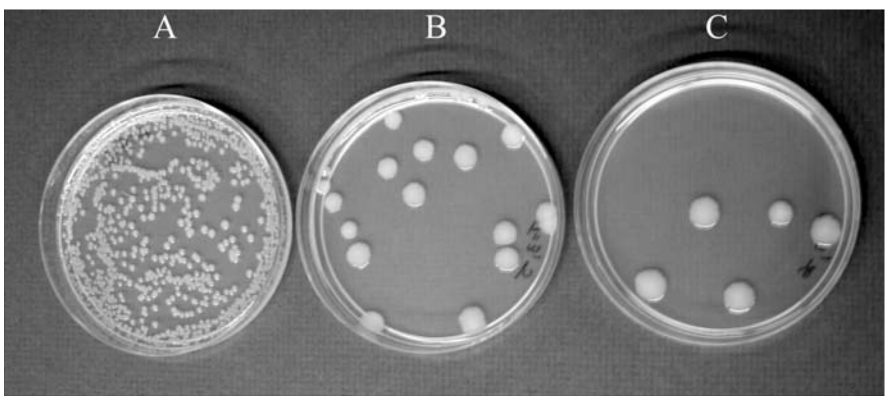


Figure 1-4 Example of a dilution series of *E. coli* plated at three dilutions. Dilutions decrease from left to right. Here, plate A is the one which should be counted (Photo courtesy K.L. Josephson).

- Change pipette tips to prevent contamination
- Label the Petri plate bottoms, not the tops

DO NOT:

- Place ethanol jars next to Bunsen flames since it may cause a fire
- Leave Petri plates exposed without lids since this will allow for bacterial contamination

1.5. POTENTIAL HAZARDS

- Fires that result from ethanol jars
- Inhalation of ethanol

1.6. EXAMPLE CALCULATION OF MEAN GENERATION TIME

Following a dilution and plating experiment, the following data was obtained. At the beginning of exponential growth designated here as time t = 0 initial concentration of bacterial cells is 1000/ml

At time t = 6 hours, the concentration of cells is 16,000/ml

Now, $X = 2^n X_o$

Where: X_o = initial concentration of cells = 1000/ml
X = concentration of cells after time t = 16,000/ml
n = number of generations

$\therefore 16{,}000 = 2^n \times 1000$

$\therefore 2^n = 16$

$\therefore \log_{10}2 = \log_{10} 16$

$\therefore n(0.301) = 1.204$

$$\therefore n = \frac{1.204}{0.301} = 4$$

∴ Four generations in 6 hours

∴ **Mean Generation Time = 6/4 = 1.5 hours**

1.7. QUESTIONS AND PROBLEMS

1. From the following data calculate the mean generation time

   At the beginning of exponential growth when time t = 0, initial cell concentration = 2500 per ml

   At time t = 8 hours cell concentration = 10,000 per ml

2. What potentially causes a lag phase during growth of a bacterial broth culture?

3. What potentially causes the death phase of a bacterial broth culture.

4. What are some of the potential errors associated with dilution and plating?

1.8. REFERENCE

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Soil Moisture Content Determination

EXPERIMENT 2

2.1. OVERVIEW

Objective: ***To determine the soil moisture content on a dry weight basis.***

- Weigh moist soil
- Dry at 110°C
- Re-weigh oven dry soil
- Calculate moisture content on a dry weight basis

2.2. THEORY AND SIGNIFICANCE

The moisture content of a soil is important for many reasons. First, all soil microbes require moisture for existence. In addition, soil moisture content controls the amount of pore space occupied by water and air, thereby determining whether the soil environment is aerobic or anaerobic. The moisture content of a soil can dramatically alter the physical appearance and properties of a soil. Figure 2-1 shows a Pima clay loam soil with varying amounts of soil moisture. Finally, the extent of soil moisture influences the transport of soluble constituents through the profile, into subsurface environments (Maier et al., 2000). All soil microbes require water or moisture, and are surrounded by water films from which they obtain nutrients and excrete wastes (Maier et al., 2000).

Most of the analyses performed in this section of the manual will involve standardization of final results on a dry weight soil basis. This is important as soils vary widely in moisture content both between soils and for any given soil over time, whereas the dry weight of a soil is constant over time.

Coarse-textured soils high in sand which contain no colloidal sized particles such as clay, contain water that is easily removed from the soil by drying. Water contained within the minerals (structural water) is very small in quantity, and is only removed at high temperature.

In contrast to coarse particles, colloidal particles, such as clays, contain both structural water and significant amounts of adsorbed water. This adsorbed water is intimately associated with the mineral structure of the particle and may be as difficult to remove as the structural water. In addition, water held adsorbed to clays is less available to soil microbes. Therefore, drying under elevated temperature is usually employed to remove free and structural water. The optimal range of temperature for drying soil with respect to removing water is between 165 and 175°C, but the problems associated with oxidation or decomposition of organic matter require the compromised



Figure 2-1 Pima clay loam soil with increasing soil moisture from left to right. The sample on the far left is completely dry, whereas the one on the far right is saturated with water. (Photo courtesy K.L. Josephson).

temperature of 100 to 110°C. Soils high in volatile organic matter may require lower drying temperatures.

In microbial analyses, soil moisture content is usually reported as the gravimetric moisture content, θ_g, which, as the name implies, is the mass of water per unit mass of oven dry soil. It is defined as:

$$\theta_g = \frac{m - d}{d} \tag{2-1}$$

where: m is the moist soil mass prior to drying, and
d is the dry mass of the same soil after drying in an oven.

On the other hand, soil moisture content as determined by some field instruments, such as a neutron probe, is often expressed as the volumetric water content, θ_v, which is the volume of water per unit volume of soil. It is related to θ_g by the following equation:

$$\theta_v = \frac{p_b}{p_w}\theta_g \tag{2-2}$$

where: p_b is the soil bulk density (commonly 1.4 to 1.6 $g\,cm^{-3}$, and
p_w is the density of water (1.0 $g\,cm^{-3}$).

However, the availability of water to microorganisms and plants alike is very much a function of how tightly the water is bound to the soil particles. Often the term "field-capacity" has been used to describe the water content of a wetted soil profile in the field, after the soil has been allowed to drain for two days (Jury et al., 1991). Soils at "field capacity" are generally optimal for aerobic soil microbes since oxygen and moisture are readily available. Water in a sandy soil at a given moisture content is much more available than a clayey soil at the same moisture content, due to the strong adsorption of

water to colloidal clay material. However, pore space and water holding capacity are greater in a clayey soil than in a sandy soil.

2.3. PROCEDURE

First Period

Materials
fresh soil
gravity convection oven preheated to 105°C
benchtop balance (±0.01 g)
2 aluminum weighing dishes per soil type

1. For each soil:
   a) Weigh 2 aluminum dishes
   b) Fill each dish with moist soil and re-weigh
   c) Dry the soil and dishes in an oven for at least 24 hrs at 105°C.

Second Period

Materials
soils from Period I
desiccator
benchtop balance (±0.01 g)

1. a) Remove the dishes from the oven, allow to cool in a desiccator, and weigh.
   b) Record the weight of the dry soil + dish.

2. Calculate the gravimetric moisture content of each of the soil samples using Eq. 2-1.

3. Report the average moisture content from the two replicate values.

2.4. TRICKS OF THE TRADE

DO:

- Label aluminum dishes in a manner that will survive heating at 110°C
- Weigh out at least 20–30 g of soil
- Dry soil for at least 24 h

DO NOT:

- Overfill the dishes, so that you spill soil on the way from the balance

2.5. POTENTIAL HAZARDS

DO:

- Wear gloves or use tongs when handling material from the oven

2.6. EXAMPLE CALCULATIONS

All of your future results will be reported on a dry weight basis, so it is worth your while to spend some time understanding the equation for the determination of the moisture content. You will additionally need the equation to manipulate the moisture content of your soils.

1. One hundred grams of moist soil has 50% moisture on a dry weight basis. How much dry soil is there in this soil sample?

Solution

$$\theta_g = \frac{m-d}{d}$$

$$\theta_g = 0.5 \qquad m = 100 \qquad d = ?$$

$$0.5 = \frac{100-d}{d}$$

$$1.5d = 100$$

Amount of dry soil, *d* = 66.6 g

2. How much water must be added to 100 g of a moist soil at an initial moisture content of 10%, so that the final soil moisture content is 15%?

Solution

$\theta_g(\text{Initial}) = 0.10$
$m(\text{Final}) = 100$
$d = ?$

$$0.1 = \frac{100-d}{d}$$

$1.1\,d = 100$
$d = 90.9$

$\theta_g(\text{Final}) = 0.15$
$d = 90.9$
$m(\text{Final}) = ?$

$$0.15 = \frac{m_{\text{final}} - 90.9}{90.9}$$

$M_{\text{final}} = 13.64 + 90.9$
$m_{\text{final}} = 104.54$

Therefore: Amount of water that must be added

= 104.54 − 100
= 4.54 g water

3. How much soil initially at 25% moisture content must be weighed out, so that following addition of water there is 100 g of a final soil sample at a moisture content of 40%?

<u>**Solution**</u>

$\theta_g(\text{Final}) = 0.4$
$m(\text{Final}) = 100\,\text{g}$
$d = ?$

$$0.4 = \frac{100 - d}{d}$$

$1.4d = 100$
$d = 71.4\,\text{g}$

$\theta_g(\text{Initial}) = 0.25$
$d = 71.4\,\text{g}$
$m(\text{Initial}) = ?$

$$0.25 = \frac{m - 71.4}{71.4}$$

$m = 17.85 + 71.4\,\text{g}$
$m = 89.25\,\text{g}$

Therefore: 89.25 g of the soil at 25% moisture should be weighed out and 10.75 g of water added to it.

2.7. QUESTIONS AND PROBLEMS

1. How does soil moisture content affect the activity of aerobic and anaerobic soil microorganisms?

2. How does soil moisture affect transport of soluble pollutants?

3. One hundred grams of a moist soil is initially at a moisture content of 33%. How much water must be added to result in a final soil moisture of 40%?

4. For the soil in question 2, how much glucose must be added on a dry weight soil basis if the glucose amendment is 10%?

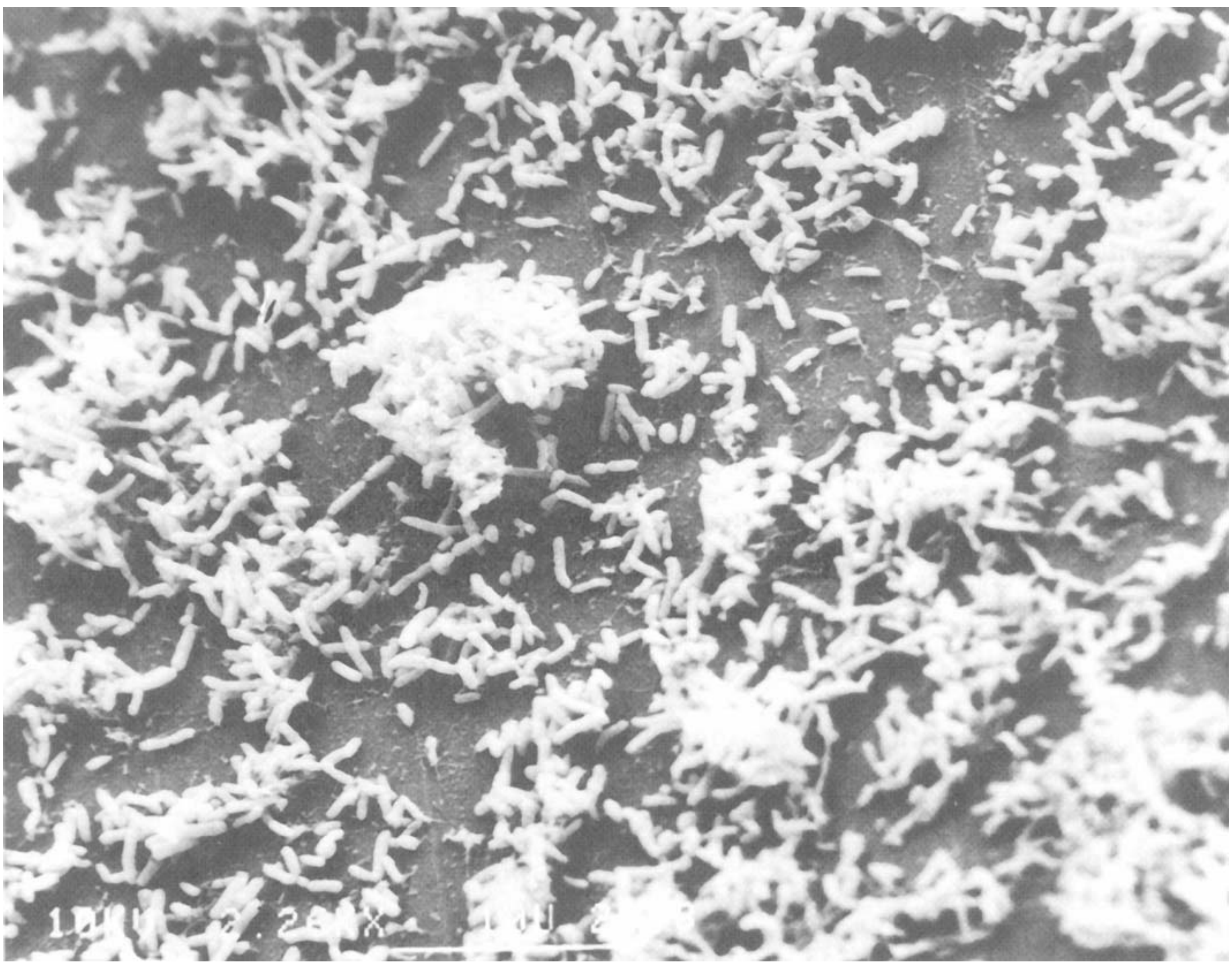
2.8. REFERENCES

Jury, W.A., Gardner, W.R., and Gardner, W.H. (1991) *Soil Physics*. 5th edition. John Wiley & Sons, Inc., New York.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

SECTION TWO

Examination of Soil Microorganisms Via Microscopic and Cultural Assays



Scanning electron micrograph of bacteria

Contact Slide Assay

EXPERIMENT 3

3.1. OVERVIEW

Objective: ***To utilize a microscope to view soil microbes and their relationship to each other and soil particles.***

- Adjust soil moisture to a value close to "field capacity" (value provided by instructor)
- Insert glass slides into a beaker of moist soil
- Incubate for one week
- Remove slides, stain with phenolic Rose Bengal
- View under microscope

3.2. THEORY AND SIGNIFICANCE

The ability to view soil microbes *in situ* is important since it allows students to view the interrelationships between soil microbes and their interactions with soil particles. However, it is difficult to observe colloidal size microbes that exist within soil. A technique developed back in the 1930s is still a valuable learning tool today. This is the contact slide or buried-slide technique of Rossi et al. (1936), which is a simple technique for qualitatively assessing the spatial relationships between soil microorganisms. Although it is not reliable enough to quantify soil microorganisms as the original authors had intended, it is useful to illustrate the orientation of soil organisms to one another and to soil particles. It also allows students to see bacteria, actinomycetes and fungi, perhaps for the first time, through the use of a microscope (Maier et al., 2000). The technique involves burying a glass slide in soil for a defined period of time (Figure 3-1). Nutrient amendments, such as the carbon source glucose and the nitrogen source ammonium nitrate, encourage the rapid proliferation of heterotrophic microorganisms.

After removing the slide from within the soil, the slide is fixed with acetic acid and stained to provide contrast, as the often colorless organisms would otherwise not be visible under a microscope. Viewed under a microscope, soil bacteria, actinomycetes, and fungi can be seen growing on soil particles, in pure colonies on the slide, and in juxtaposition to each other, often with bacteria lining the fungal hyphae. Spore formation by actinomycetes or fungi can also be observed. Examples of what may be seen are shown in Figures 3-2 and 3-3.



Figure 3-1 Examples of soil microcosms with inserted buried glass slides (Photo courtesy K.L. Josephson).

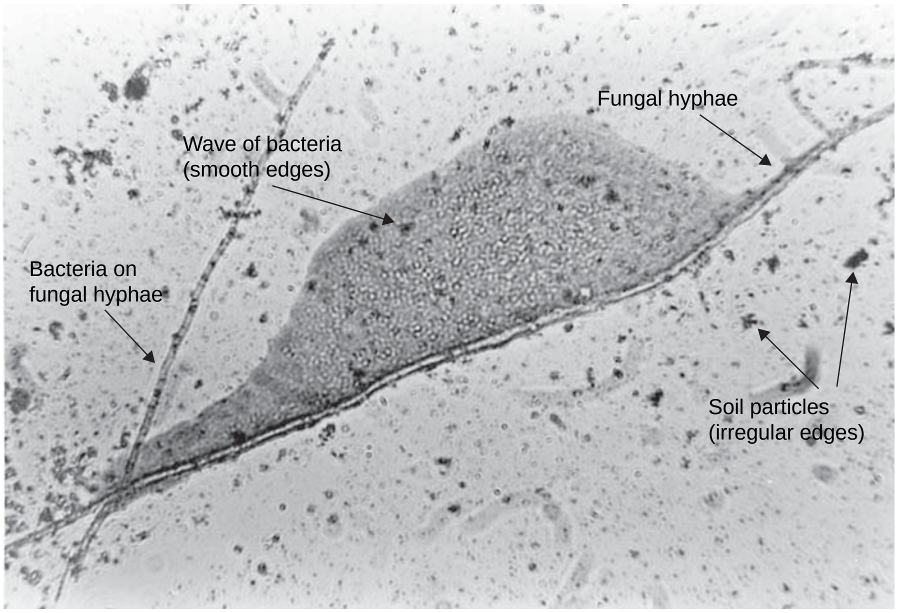


Figure 3-2 Contact slide images using the 100× objective lens (Photo courtesy W.H. Fuller).

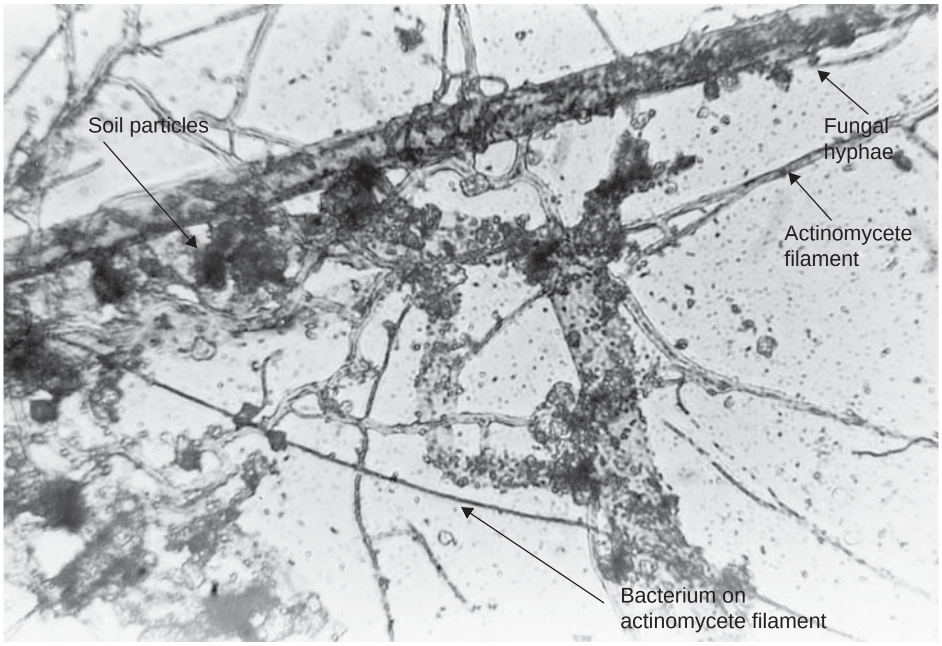


Figure 3-3 Contact slide images using the 100× objective lens (Photo courtesy W.H. Fuller).

3.3. PROCEDURE

First Period

Materials
300 g of each soil
1% glucose
NH_4NO_3
2 polystyrene cups for each soil type, volume $\simeq$ 250 ml
label tape and pens
plastic wrap
4 microscope slides for each soil type
rubber bands
weighing paper
deionized water in a wash bottle
analytical balance, and benchtop balance (±0.01 g)
graduated cylinder

1. Weigh out 150 g portions of each soil into two cups, recording the mass of the soil you added to each cup. Label one cup as "treatment" and the other as "control." A 100 g sample of soil should be used for soils high in organic matter, as they are less dense than mineral soils.

2. Calculate the amount of moisture necessary to alter the moisture content of the soil samples to the moisture content specified by your instructor. This soil moisture content is often close to field capacity. Measure out this much distilled water with a graduated cylinder and add it to each of two vials. Label one vial "treatment" and the other "control."

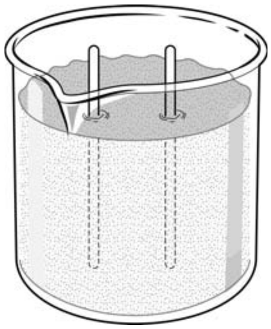


Figure 3-4 Position of the slides in the tumbler containing soil.

3. Amend the water in the treatment vial with enough glucose for a final soil glucose concentration of 1% (w/w) on a dry weight basis in the treatment soil above. Also add 200 mg of NH_4NO_3 to the treatment vial. Stir to dissolve the amendments. Do not amend the control vial.

4. Mix the contents of the treatment and control vials into their respective cups by adding the liquid to the soil in small aliquots, and mixing with a spatula after each moisture addition. For heavy textured clay soils avoid mixing as this will "puddle" the soil.

5. For each cup, label two clean microscope slides, designating the soil and treatment for that slide. There will be two slides for each cup. Insert each slide vertically into its respective cup, leaving 2 cm of each slide projecting above the soil surface (see Figures 3-4). Do not force the slides as they will break.

6. Cover the cups with plastic wrap, securing with a rubber band. Puncture the wrap or foil several times with a probe to allow air in and yet preclude excessive evaporation of moisture. Weigh each cup. Incubate the soil-filled cups at room temperature in a designated incubator for one week.

Second Period

Materials
incubated cups from Period 1
40% (v/v) acetic acid
phenolic Rose Bengal stain
staining racks with a pan to catch excess stain
protective goggles
microscopes
immersion oil
paper towels

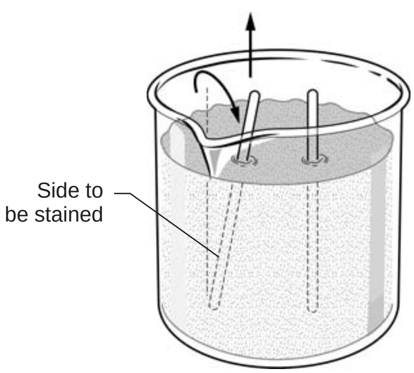


Figure 3-5 Withdrawing a slide from the soil. Gently tilt the slide to one side before pulling straight up so as not to disturb the organisms on the upper face.

1. Re-weigh the cup and calculate the soil moisture at the time of slide removal.

2. Remove the two slides from each cup after seven days by pressing each slide to an inclined position and withdrawing in a manner such that the upper face of the slide is not disturbed. Mark and identify the side to be stained (see Figures 3-5 and 3-6).

3. Gently tap the slide on the bench top to remove large soil particles from the slide surface. Clean the lower face with a damp paper towel and dry the slide at room temperature.

4. Wearing protective goggles, immerse the slide in 40% (v/v) acetic acid for 1–3 min under a fume hood, holding the slide with forceps.

5. Wash off the excess acid under a gentle stream of water, and cover the surface with phenolic Rose Bengal from a dropper bottle, supporting the slide on a staining rack over a container to catch the excess stain.



Figure 3-6 Example of how the slide plus accompanying soil should look following removal of the slide from the soil (Photo courtesy K.L. Josephson).

Be careful not to wash with such force as to remove microorganisms from the slide surface.

6. Stain for 5–10 minutes, *but do not permit the slide to become dry*. Add more stain as needed.

7. Gently wash the slide to remove excess stain. Dry and examine the slide microscopically using the oil immersion objective. Compare what you see with Figure 3-2 and Figure 3-3.

3.4. TRICKS OF THE TRADE

DO:

- Label each beaker
- Weigh beakers before and after incubation to track soil moisture loss
- Remove almost all soil from the slides prior to staining. You should just be able to see specks of soil, but not large lumps of soil

<u>DO NOT</u>:

- Mix the soil if it is high in clay content ("puddle" soil)
- Forget to add the slides to the beakers (students have been known to forget!)
- Break the slides as you remove them from the beakers

3.5. POTENTIAL HAZARDS

<u>DO</u>:

- Handle acetic acid only in a fume hood and wear goggles.
- Use a fume hood and vinyl gloves when working with phenol.

<u>DO NOT</u>:

- Breathe in acetic acid or phenol fumes.

3.6. QUESTIONS AND PROBLEMS

1. Describe the size and shape of bacterial cells, filaments and spores of fungi and actinomycetes.

2. Note evidence of colony formation and the relationships of organisms to each other and to soil particles. Make sketches showing typical fields for each soil/treatment combination. Label each drawing with the soil and treatment it depicts.

3. Describe qualitative differences in the microbial populations between the different soils for each treatment, i.e., unamended and amended with glucose and NH_4NO_3.

4. Were there quantitative differences in microbial population densities between the soils or between the treatments? Speculate as to why there were or were not any differences between soils and treatments.

5. How did you distinguish fungi from actinomycetes?

6. Discuss the usefulness of the method. Indicate how the results have influenced your concept of the nature of the development of microorganisms in soil and the significance of numbers of cells of microorganisms as found in published literature.

7. How were microorganisms oriented with respect to each other and soil particles?

8. Was there competition for space between microorganisms?

9. What appears to have been the limiting factor for microbial growth and activity in soil?

3.7. REFERENCES

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Rossi, G., Riccardo, S., Gesue, G., Stanganelli, M., and Wang, T.K. (1936) Direct microscopic and bacteriological investigations of the soil. *Soil Science* **41**, 52–66.

Filamentous Fungi

EXPERIMENT 4

4.1. OVERVIEW

Objective: ***To isolate, observe, and quantify filamentous soil fungi using dilution and plating techniques.***

- Adjust moisture content of soil to a value close to field capacity (value provided by instructor)
- Adapt soil to new moisture content by incubating for 1 week
- Dilute soil and serially plate dilutions via "pour plates"
- Incubate plates for one week
- Count fungal colonies and identify different fungal genera via microscopic examination

4.2. THEORY AND SIGNIFICANCE

Fungi are heterotrophic eukaryotic organisms, and with the exception of yeasts, are aerobic. They are abundant in surface soils and important for their role in nutrient cycling and decomposition of organic matter and organic contaminants (Maier et al., 2000). White rot fungi (*Phanerochaete chryosporium*), for example, are known to degrade aromatics (Hammel, 1995).

Since soils generally contain millions of fungi per gram, normally a dilution series of the soil is made by suspending a given amount of soil in a dispersing solution (often deionized water), and transferring aliquots of the suspensions to fresh solution until the suspension is diluted sufficiently to allow individual discrete fungal colonies to grow on the agar plates.

After inoculation on several replicate agar plates, the plates are incubated at an appropriate temperature and counted after they have formed macroscopic fungal colonies (Figure 4-1). Because the assumption is that one fungal colony is derived from one organism, the term **colony forming units** (**CFUs**) is used in the final analysis, with the results expressed in terms of CFUs per gram of oven dry soil.

Values for culturable fungal counts from a fertile soil have been reported as around 10^6 fungal "propagules" (spores, hyphae, or hyphal fragments) per gram of dry soil (Pepper et al., 1996).

Figure 4-2 describes a dilution and plating protocol procedure. Beginning at step 1, a 10-fold dilution series is performed. A 10-fold series is very common as the calculations for the determinations of the organism count is very simple. Here, 10 g of moist soil is added to 95 ml (solution A) of deionized water and shaken well to disperse the organisms. The reason that 10 g of soil

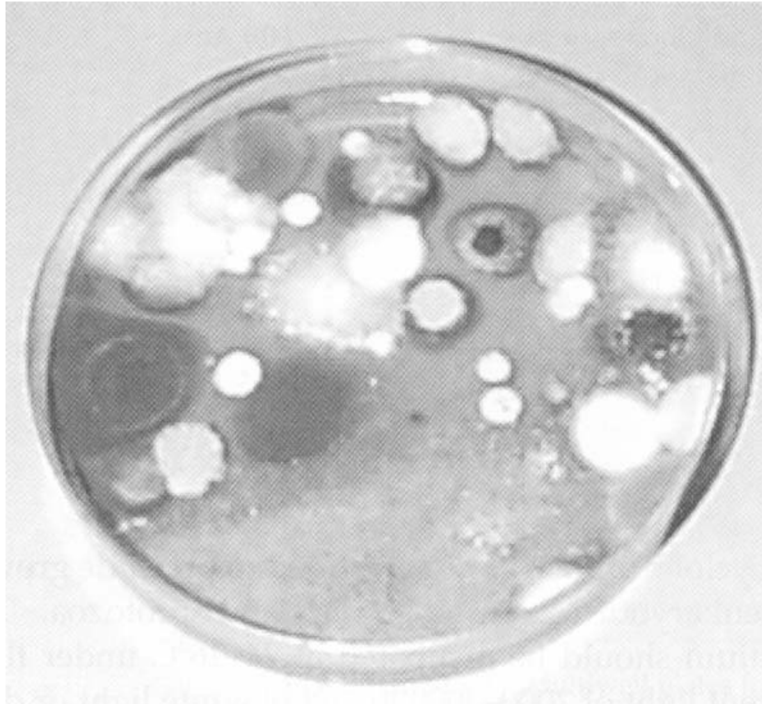


Figure 4-1 An example of a fungal pour plate, with macroscopic colonies (Photo courtesy K.L. Josephson).

are used is that 10 g of soil occupies approximately 5 ml. Thus, we have 10 g of soil in 100 ml total volume, thereby forming a 1 : 10 w/v dilution.

Next, 1.0 ml of suspension is removed from the bottle and added to a tube (B) containing 9.0 ml of the same dispersion solution as in A. The tube is capped and vortexed. Working diligently, the dilution series is continued to the highest desired dilution (tubes C, D, and E). The three most diluted suspensions are plated. Three different dilutions (tubes C, D, and E) are plated so as to increase the chance of obtaining a dilution that will result in a countable number of organisms. (See Figure 4-3 for a plated dilution series.)

Here, pour plates are utilized for the plating procedure. The dilution of interest is vortexed and 1.0 ml of suspension is removed from the tube and added to each of two sterile Petri dishes. Before the soil particles in the inoculum can settle, pour plates are made (step 3a). Here, a suitable agar is poured into the plate with the one ml of inoculum. The agar is at a temperature warm enough to keep the agar fluid, but cool enough not to kill the organisms or destroy any heat-sensitive amendments to the agar (e.g., antibiotics).

Then the plate is *gently* swirled (step 3b) to distribute the agar and inoculum across the bottom of the plate *(without splashing agar on the sides or lid of the dish)*. Finally, the agar is allowed to solidify, and the plates are incubated upside down to prevent condensation from falling on the growing surface of the agar (step 4). Counting takes place after an incubation period suitable for the organism(s) of interest (often 5–7 days).

Using pour plates is useful for fungi since fungi can rapidly grow through agar but bacteria cannot. Other types of plating are possible. Spread plating

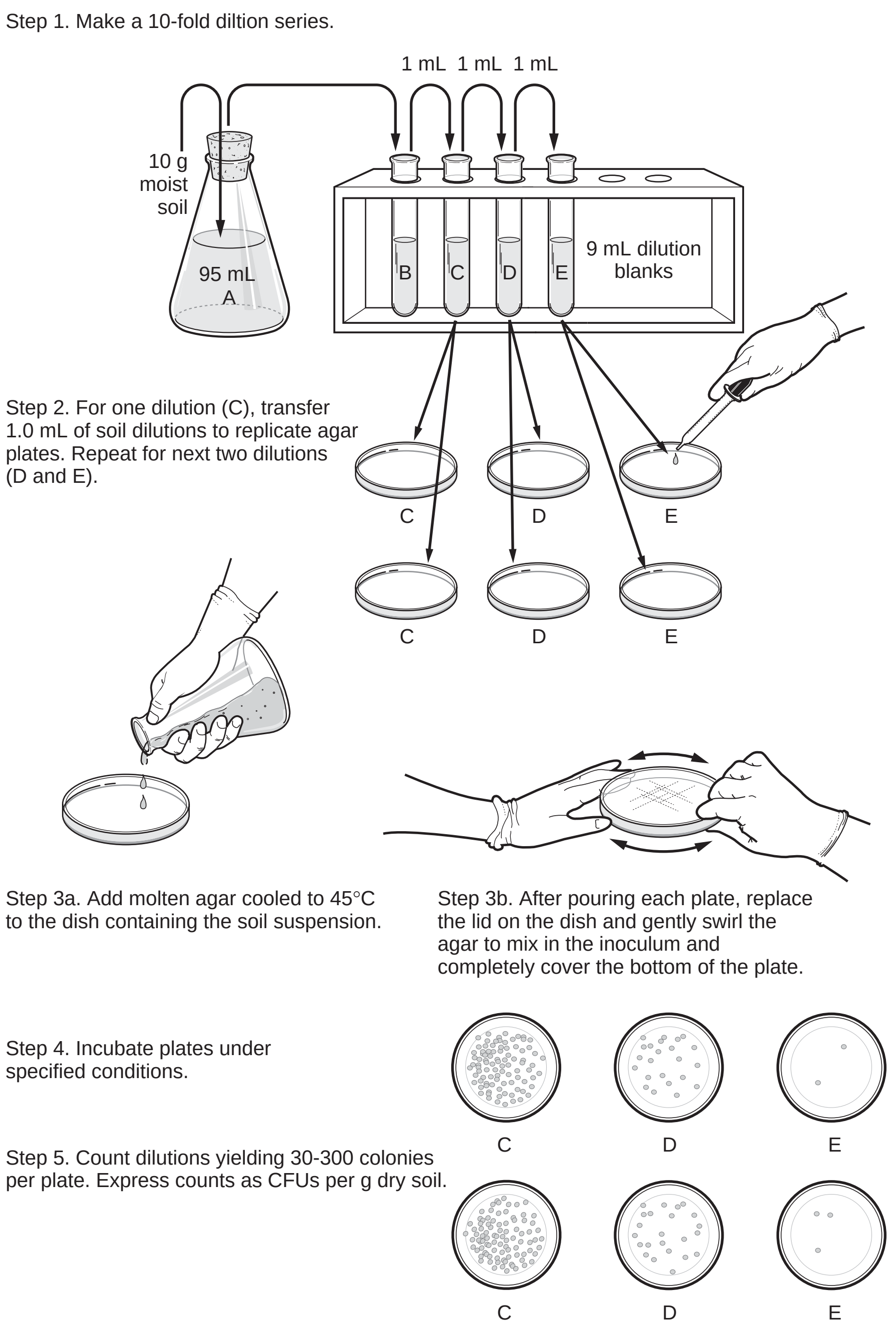


Figure 4-2 Schematic showing the procedure for culturable heterotrophic plate counts of filamentous fungi.

will be used in the next exercise. As the described technique involves working and incubating in the open air, aerobic and facultative anaerobic organisms are enumerated. Obligate anaerobes are not enumerated.

Culturable heterotrophic plate counts have been in use for enumerating organisms since the nineteenth-century. They continue to be used today as they are inexpensive to perform, require little labor, are quick, and are fairly reproducible. However, they do suffer from a number of errors which must be considered when evaluating the results.

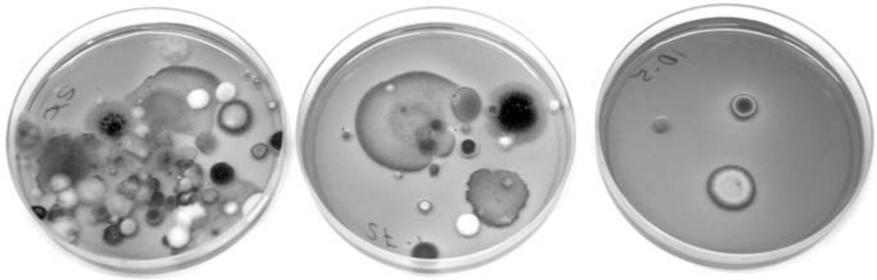


Figure 4-3 Macroscopic fungal colonies that result from incubation following dilution and plating of soil. (Photo courtesy K.L. Josephson).

ERRORS AND ASSUMPTIONS

1. The assumption of one organism per colony is rarely satisfied as several cells associated with a soil particle may give rise to one colony. Microscopic direct counts do not make this assumption.
2. Errors in diluting the soil can arise from either particles not dispersing entirely (less dilution occurs) or from particles settling out of solution prior to the next dilution (more dilution occurs).
3. Only a small fraction of organisms will grow on a given medium. Microscopic direct counts do not make this assumption. Therefore direct counts are often referred to as total counts, which will typically be 1 to 2 orders of magnitude greater than the culturable count (Maier et al., 2000).
4. As soil is a heterogeneous medium, biological variability may be high even between adjacent areas of soil.
5. Heavily sporulating organisms (including many fungi) are often overemphasized.

4.3. PROCEDURE

First Period

Materials
25 g fresh soil of each soil type
deionized water
25-ml pipette
pipette bulb
1 plastic vial for each soil type
rubber bands
plastic wrap
benchtop balance (±0.01 g)

1. Calculate the amount of water that needs to be added to 25 g of soil to amend the soil moisture content to a value specified by your instructor. Next, add that amount of moisture (deionized water) to 25 g of soil that was previously weighed into the containers provided.

2. Cover the containers with plastic wrap and puncture the film several times with a probe to allow aeration during incubation. Secure the film with a rubber band. Weigh the soil and wrap; you will need this information to calculate moisture loss from the soil during incubation at room temperature for one week.

Second Period

Materials
incubated soils from Period 1
1 sterile, 95 ml water blank per soil type
3 sterile, 9 ml water blanks per soil type
150-ml Rose Bengal agar for each soil type
filter-sterilized streptomycin solution to bring the agar to 30 µg ml^{-1}
9 sterile Petri dishes per soil type
6 sterile, 1 ml pipettes per soil type
deionized water
1 test tube rack
pipette bulb
pan for collecting excess agar
vinyl gloves
marking pens
benchtop balance (±0.01 g)
vortex
water bath at 45°C to keep agar molten prior to pouring

1. Weigh each of the soil samples with the wrap and rubber band and record the weights. The weight loss is due to moisture loss. Thus the actual soil moisture at the time of plating can be calculated. Prepare a 1/10 dilution series of your soils as shown in Figure 4-2.

2. This will give you dilutions of 10^{-1} (bottle A), 10^{-2} (tube B), 10^{-3} (tube C), 10^{-4} (tube D) and 10^{-5} (tube E) g soil ml^{-1} suspensions.

3. Prepare two plates for each of these dilutions, for example 10^{-2}, 10^{-3}, and 10^{-4} (tubes B, C, and D), by adding 1.0 ml of each dilution to three separate sterile Petri plates for each soil (6 plates for each soil). Your final effective dilutions will be 10^{-2}, 10^{-3}, and 10^{-4} g soil per plate. The medium is Rose Bengal-streptomycin agar. Both the Rose Bengal and streptomycin inhibit bacterial growth. For very fertile soils where soil microbial populations are high, the chosen dilutions should be higher i.e., 10^{-3}, 10^{-4}, and 10^{-5}. Your instructor will choose the dilutions for your soil.

4. Incubate plates at room temperature for one week.

Third Period

Materials
incubated plates from Period 2
lactophenol mounting fluid
pressure or transparent tape
dissecting probe
forceps
microscope slides
immersion oil
microscope
fungal identification key

1. Make colony counts at one and only one dilution of each soil. The plates that are counted should have discrete countable colonies. Overgrown plates should not be counted. Likewise, plates with <10 colonies should not be counted. Note and describe the cultural characteristics of three different colonies. Examine colonies with the low power objective of the microscope.

2. Prepare pressure tape (transparent tape) mounts on slides for detailed microscope study using the following procedure:
   - Deposit a drop of lactophenol mounting fluid at the center of a clean glass slide.
   - Cut a strip of clear cellophane tape about 3 cm long from the stock roll. To avoid contaminating the adhesive surface, use forceps when handling the tape. A dissecting needle will aid in freeing the tape from the forceps.
   - The adhesive side of the tape is applied to the surface of a sporulating fungus colony. Take care to avoid excessive pressure on the tape or too dense a mass of hyphae and spores will be collected.
   - Remove the tape from contact with the fungus colony and apply it, adhesive side down, to the drop of mounting fluid on the glass slide. Rub the tape gently with a smooth, flat instrument to express air bubbles.

3. Examine the fungi microscopically under the oil immersion objective.

4. Also examine directly the mycelium of "fast spreading" fungi on the plate, under the 10× objective of the microscope.

5. Identify three different fungal genera using the supplied fungal identification key (Figure 4-4). Describe and illustrate the fruiting bodies of these fungi via sketches.

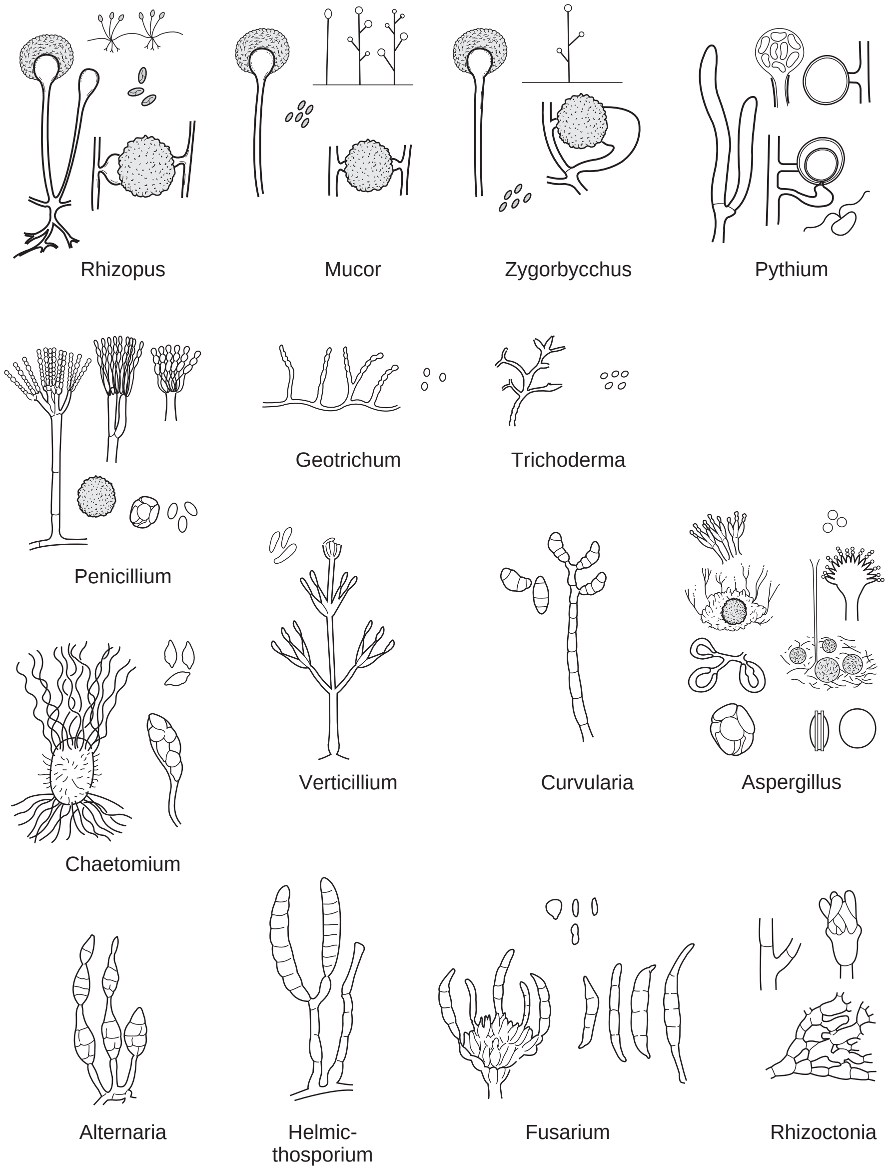


Figure 4-4 Illustrations of fruiting bodies of fungi often isolated from soil.

4.4. TRICKS OF THE TRADE

DO:

- Add water to a clayey soil without stirring, to avoid “puddling” the soil during plating
- Prepare plates from three successive dilutions to ensure getting a countable number of colonies
- Change pipettes appropriately when plating different dilutions
- Gently swirl the media during plating to distribute the soil inoculum without getting media on the Petri dish lid
- Check to make sure the agar has hardened before inverting the plates
- Count only one dilution of the soil

DO NOT:

- Let soil particles settle before removing an aliquot during preparations of soil dilutions, i.e., swirl dilutions, then remove aliquot immediately
- Spill from dilution tubes—the caps are probably not tight
- Forget to add the streptomycin to the media
- Add the media to the soil inoculum when it is too hot, since this will kill microbes
- Allow the media to cool too much since it will solidify, i.e., media temperature should be comfortable to the touch
- Allow excess media to solidify in flasks as this makes clean up difficult
- Press too hard on the tape mounts as this will destroy fungal fruiting bodies

4.5. POTENTIAL HAZARDS

DO:

- Use gloves when working with streptomycin
- Be careful with hot agar

4.6. CALCULATIONS

Tabulate all results as illustrated below, including individual plate counts and mean counts. Calculate the total number of filamentous fungi (as colony forming units (CFUs)) per gram dry weight of soil. Compute the sample mean, sample standard deviation, and the coefficient of variation.

DILUTION AND PLATING CALCULATIONS

A 10-gram sample of soil with a moisture content of 20% on a dry weight basis is analyzed for viable culturable bacteria via dilution and plating techniques. The dilutions were made as follows:

Process		Dilution
10 g soil →	95 ml saline (solution A)	10^{-1} (weight/volume)
1 ml solution A →	9 ml saline (solution B)	10^{-2} (volume/volume)
1 ml solution B →	9 ml saline (solution C)	10^{-3} (volume/volume)
1 ml solution C →	9 ml saline (solution D)	10^{-4} (volume/volume)
1 ml solution D →	9 ml saline (solution E)	10^{-5} (volume/volume)

1 ml of solution E is pour plated onto an appropriate medium and results in 200 bacterial colonies.

$$\textbf{Number of CFU} = \frac{1}{\text{dilution factor}} \times \text{number of colonies}$$

$$= \frac{1}{10^{-5}} \times 200\,\text{CFU/g moist soil}$$

$$= 2.00 \times 10^{7}\,\text{CFU/g moist soil}$$

But, for 10 g of moist soil,

$$\text{Moisture content} = \frac{\text{moist weight} - \text{dry weight (D)}}{\text{dry weight (D)}}$$

Therefore,

$$0.20 = \frac{10 - D}{D} \quad \text{and}$$

$$D = 8.33\,\text{g}$$

Number of CFU per g dry soil $= 2.00 \times 10^7 \times \dfrac{1}{8.33} = 2.4 \times 10^7$

FORMULAE

Formula D-3 Formula for calculating the sample mean of a data set.

$$\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n}$$

$\bar{y}$ = sample mean
i = index
y_i = the value of data point number i
n = the total number of data points in the set

Formula D-5 Formula for calculating the sample standard deviation of a data set.

$$s_y = \left(\frac{\sum_{i=1}^{n} (y_i - \bar{y})^2}{n - 1} \right)^{\frac{1}{2}}$$

s_y = sample standard deviation
i = index
$\bar{y}$ = sample mean
y_i = the value of data point number i
n = the total number of data points in the set

Formula D-2 Formula for calculating coefficient of variation.

$$CV = \frac{s_y}{\bar{y}} \times 100$$

CV = coefficient of variation
s_y = sample standard deviation
$\bar{y}$ = sample mean

4.7. QUESTIONS AND PROBLEMS

1. Make a sketch of two genera of fungi you have identified.

2. What is the influence of pH on the abundance of fungi in soil? Why?

3. Would fungi tend to be more dominant in desert soils or prairie grass land soils? Why?

4. Were there differences in fungal populations in different soils?

5. Is dilution and plating a good method for getting absolute numbers of fungi in soil? Why or why not?

6. Identify two major benefits and two major hazards of soil fungi.

7. What is the meaning of standard deviation? Discuss the relevance of statistics to studies of the microbial population of soil.

4.8. REFERENCES

Hammel, K.E. (1995) Organopollutant degradation by ligninolytic fungi. In: *Microbial Transformation and Degradation of Toxic Organic Chemicals.* L.Y. Young and C.E. Cerniglia, eds. Wiley & Sons, New York, NY.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Pepper, I.L., Gerba, C.P., and Brusseau, M.L. (1996) *Pollution Science*. Academic Press, San Diego.

Bacteria and Actinomycetes

EXPERIMENT 5

5.1. OVERVIEW

Objective: ***To isolate, observe, and quantify soil bacteria, and also to examine antibiotic resistance of selected isolates.***

- Adjust moisture content of soil to a value close to field capacity (value provided by instructor)
- Adapt soil to new moisture content by incubating for one week
- Dilute soil and serially plate dilutions via "spread plates." Plate on two sets of media, one designed for bacteria, the other more suited to actinomycetes
- Incubate "bacterial" plates for one week and "actinomycete" plates for two weeks
- Count bacterial and actinomycete populations at the end of the incubation period
- Isolate pure cultures of bacteria and actinomycetes via "streak plates"
- Perform Gram stain on pure cultures
- Test pure cultures for antibiotic resistance

5.2. THEORY AND SIGNIFICANCE

Soil bacteria are the most abundant organisms found in surface soils. These organisms are very diverse; all are prokaryotic, but the bacteria can be aerobic, anaerobic of facultatively anaerobic. In addition, there are autotrophic and heterotrophic bacteria (Maier et al., 2000). Within this prokaryotic group are the filamentous microbes known as actinomycetes. Bacteria and actinomycetes are important in nutrient cycling and degradation of organic contaminants. In addition, they interact with plants as "rhizosphere" populations in close proximity to plant roots. Finally, soil bacteria can be pathogenic to plants (*Agrobacterium tumefaciens*) and humans (*Clostridium perfringens* and *Bacillus anthracis*) (Pepper and Gentry, 2002).

The theory behind culturable heterotrophic plate counts for enumerating bacteria and actinomycetes has already been discussed in Experiment 4, Filamentous Fungi.

As in the case of the fungi, a soil sample is serially diluted and plated on agar to achieve a countable number of organisms on the plates. Figure 5-1 shows the results of a culturable heterotrophic plate count assay. Fungi are rarely a problem on bacterial plates since bacteria are so much more numerous than fungi and normally grow faster, out-competing fungi on the plate.

Both bacteria and actinomycetes are prokaryotic organisms; actinomycetes are considered to be true bacteria (Pepper et al., 1996). When grown on agar,

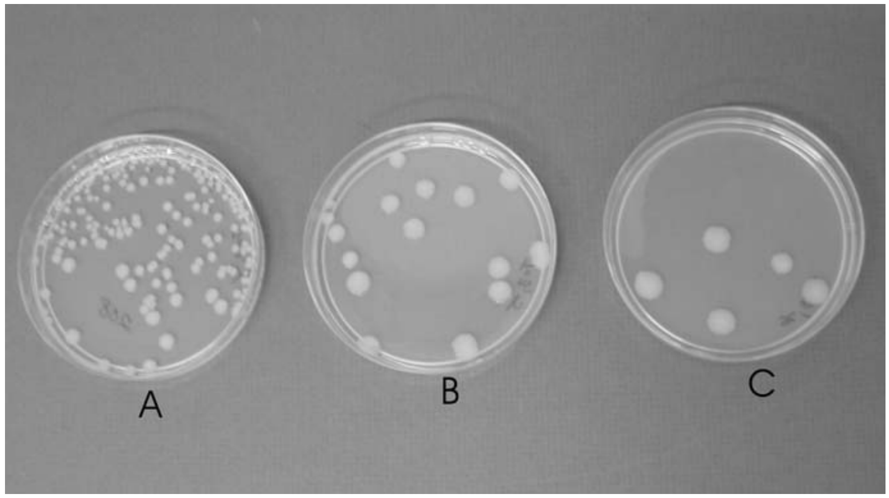


Figure 5-1 Results of a culturable heterotrophic plate count assay. The plate on the left (A) has the most bacterial colonies (lowest soil dilution). The other two plates (B & C) result from subsequent dilutions. From these three plates, plate A should be counted since it has between 30 and 200 colonies. (Photo courtesy K.L. Josephson.)

bacteria produce slimy colonies ranging from colorless to brightly colored orange, yellow, or pink colonies. In contrast, actinomycetes have a filamentous growth habit which makes it possible to visually distinguish them from the bacteria. Actinomycete colonies are chalky, firm and leathery, and will break under pressure. In contrast, bacterial colonies will smear under pressure.

Unlike bacteria, actinomycetes are relatively drought resistant and have an ecological advantage over bacteria in desert soils which tend to be dry and alkaline (Pepper et al., 1996). Therefore, drying the soil before performing culturable heterotrophic plate counts can greatly reduce the magnitude of bacterial interference. Antifungal agents, such as cycloheximide are added to control fungi.

Analyses of microorganisms in soil can be either quantitative, as in the case of culturable heterotrophic plate counts, or qualitative. Qualitative tests are more concerned with the characteristics of the organisms rather than their numbers. For example, soil samples are routinely screened by pharmaceutical companies for organisms that can produce previously unknown antibiotics. The streak plate is a common means of generating pure culture isolates.

To make a streak plate, a soil sample is serially diluted and plated to produce plates with distinct and separate colonies. After growth to a macroscopic size, colonies of interest are plated again on fresh agar by dipping a sterile inoculating loop into the colony to remove a small sample. The loop is then dragged across a fresh, sterile agar plate (Figure 5-2). As the loop is dragged, the number of organisms that are being transferred from the loop to the plate per unit length of movement is decreased (streak A in Figure 5-2).

The loop is resterilized in a flame and touched to the end of streak A to collect a small amount of organisms. Again, the loop is dragged to attenuate

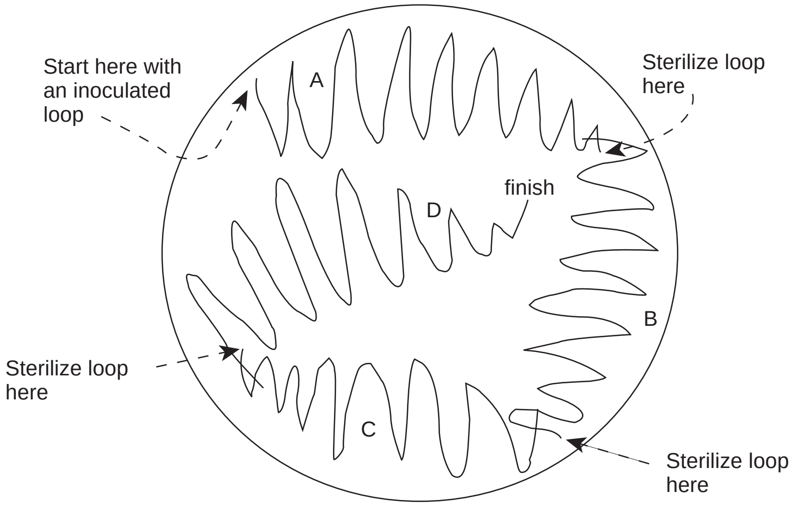


Figure 5-2 Isolating single colonies of a bacterium by making a streak plate. For successes, it is important that the loop be sterilized between streaks. (Let the loop cool so as not to kill the organisms on it.) Also, take care so as to catch the end of the previous streak, crossing it only once. Otherwise, too many organisms may adhere to the loop.

the organisms and resterilized. The procedure is successively repeated for streaks C and D. By the end of streak D, individual colonies should be apparent. Then the plates are evaluated for cultural purity. Morphologically divergent colonies in the final streak are one sign that the colony isolated from the original spread plate was formed by more than one type of organism.

A further qualitative test performed on soil microorganisms, and almost exclusively on bacteria, is the antibiotic sensitivity test. Bacteria differ widely in their resistance to various antibiotics and in the mechanisms by which the bacteria are inhibited by the antibiotics (see Box below). The genes which are responsible for resistance to antibiotics have been isolated and can be used in research to detect the organisms in soil (Pillai and Pepper, 1991). Undesired transfer of this genetic material to other bacteria can occur under certain conditions, thus spreading resistance to the antibiotic, but antibiotic resistance in nevertheless routinely used to reisolate organisms of interest.

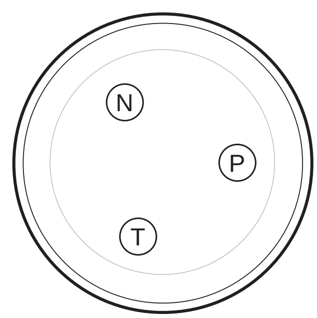


Figure 5-3 Placement of the antibiotic disks on the agar plate. In this example, N = neomycin, P = penicillin, and T = tetracycline.

The degree of antibiotic resistance of a bacterium can be qualitatively assessed by measuring the size of the inhibition zone formed on a spread plate of a pure culture to which antibiotic-containing disks of filter paper have been applied (Figures 5-3 and 5-4). The concentration of antibiotic at a given distance from one of the disks will be dependent on a number of factors, including time and distance from the disk. Thus, after a given incubation period, an antibiotic concentration gradient in the agar will have formed, radiating away from the periphery of the disk toward lower values. The off-colored, sometimes opaque, "bacterial lawn" will be absent for a larger radius around the disk which contains an antibiotic to which it is very sensitive (see Figure 5-4).

A third way of qualitatively assessing bacteria is the organisms's Gram staining property. Gram staining, either positive or negative, is based on the struc-

Box 1. Common Antibiotics Often Used in Selective Media

Name	Spectrum	Mode of Action
Chloramphenicol	Broad spectrum	Inhibits protein synthesis by binding to 50*S* ribosomal subunit
Erythromycin	Mostly gram-positive	Inhibits protein synthesis by binding to 50*S* ribosomal subunit
Tetracycline	Broad spectrum	Inhibits protein synthesis by binding to 30*S* ribosomal subunit
Streptomycin	Broad spectrum	Inhibits protein synthesis by binding to 30*S* ribosomal subunit
Polymyxin	Gram-negative bacteria, especially *Pseudomonas*	Disrupts cell membrane
Nalidixic acid	Gram-negative bacteria	Inhibits DNA synthesis
Novobiocin	Gram-negative bacteria	Inhibits DNA synthesis
Trimethoprim	Broad spectrum	Inhibit purine synthesis
Rifampicin	Gram-positive bacteria	Inhibit RNA synthesis
Penicillin	Mostly gram-positive bacteria	Inhibits cell wall peptidoglycan synthesis

ture of the bacterial cell wall. Gram staining involves treating a smear of a pure culture with an aniline dye (carbolic gentian violet or crystal violet), which is then set by the mordant iodine. In gram-negative organisms, an alcohol-based decolorizer removes the aniline dye. In gram-positive organisms, the dye remains. Following staining with the red dye safranin, **gram-negative organisms**, from which the blue aniline dye was removed, appear red. In **gram-positive organisms**, however, the blue dye remains and the cells appear blue or purple. Do note that some bacteria are known to be gram variable. That is, these organisms change their Gram stain during their life cycle. For example, *Arthrobacter* is a gram-negative rod when cells are young and a gram-positive coccus when cells are older. This can result in both blue and red cells being observed in the same stain.

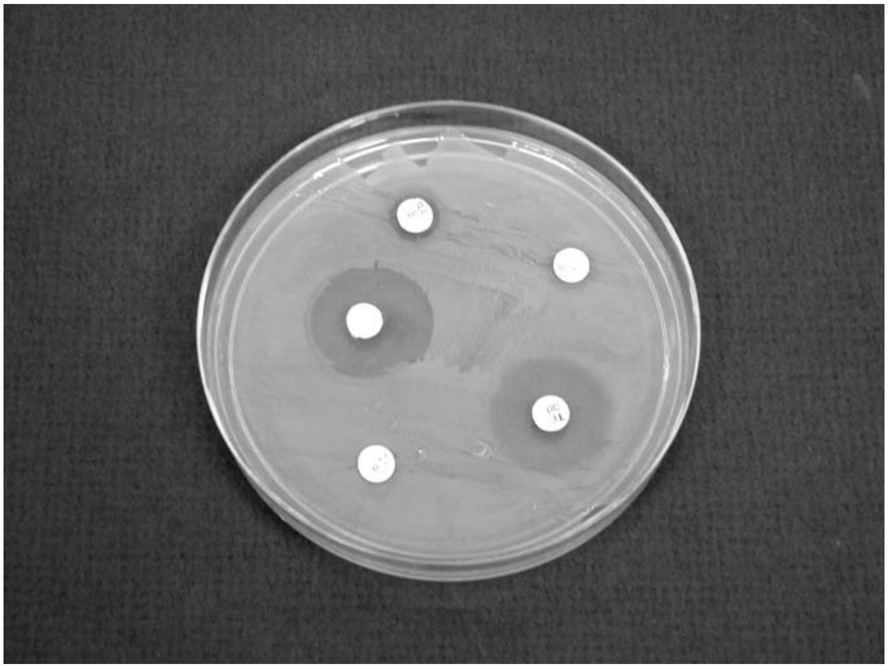


Figure 5-4 Zone of inhibition of bacterial growth on a spread plate. The inhibition is due to diffusion of antibiotics from antibiotic filter disks (Photo courtesy K.L. Josephson).

5.3. PROCEDURE

First Period

Materials
25 g fresh soil of each soil type
one plastic cup for each soil type
benchtop balance (±0.01 g)
weighing dishes
deionized water
plastic wrap
rubber bands
marking pens
dissecting probe

1. Weigh out one 25 g sample of each soil into a labeled plastic cup. Amend the soil with deionized water to the moisture content specified by your instructor. Cover the samples with plastic wrap to reduce moisture loss, and secure with a rubber band. Puncturing the wrap several times with a probe allows aeration without substantial moisture loss.

2. Weigh the samples with the plastic wrap and rubber band and record the weights. You will need these values to determine the final soil moisture content. Incubate the samples at room temperature for one week.

Second Period

Materials
incubated soils from Period 1
benchtop balance (±0.01 g)
9 peptone-yeast agar plates per soil type
9 glycerol-casein agar plates amended with cycloheximide per soil type
1 sterile, 95 ml water blank for each soil type
4 sterile, 9 ml water blanks for each soil type
10 sterile, 1 ml pipettes for each soil type
pipette bulb
1 test tube rack
glass hockey stick spreader
ethyl alcohol for flame sterilization
vortex
gas burner
pre-prepared R_2A agar plates
pre-prepared glycerol-casein agar plates

Preparation of the Plates

Agar plates already prepared will be provided to you. The medium in the plates consists of R_2A.

Preparation of Soil Dilutions for Plating

1. Re-weigh each of the soil samples including the plastic wrap covering, to allow for soil moisture calculation at the time of plating.

2. Prepare a dilution series of each of the soils (see Figure 5-5).

3. For each soil, suspend 10 g to a 95 ml water blank. Shake the suspension well.

4. Before the soil settles in the bottle, remove 1 ml of the suspension with a sterile pipette and add it to a 9 ml water blank. Vortex well.

5. Repeat the previous step three times, each time with a fresh 9 ml water blank and sterile pipette. Vortex well. This will result in dilutions of ca. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} g soil ml^{-1} (tubes A thru E).

Making Spread Plates

For Bacteria

1. Prepare two or three spread plates for each dilution 10^{-3}, 10^{-4}, 10^{-5}, as follows. After vortexing, place a 0.1 ml drop of each dilution (this will increase your effective dilution by a factor of ten) to three separate, labeled peptone-yeast agar plates. Inoculate no more than three plates before spreading, as standing will allow too much liquid to be absorbed into the agar in one spot.

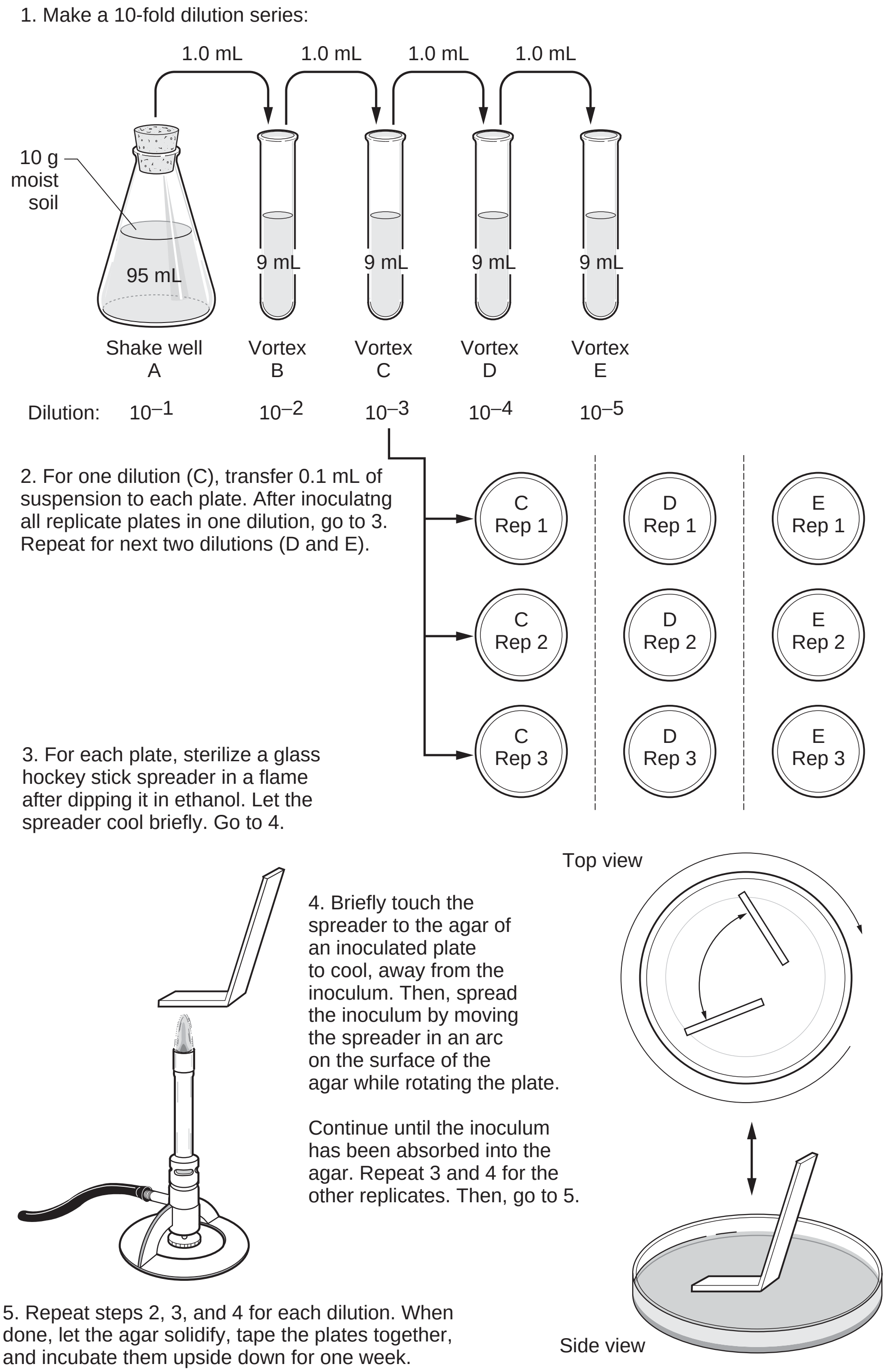


Figure 5-5 Schematic showing the procedure for viable heterotrophic plate counts of bacteria you will use in this laboratory experiment. Use tubes B, C, and D for the actinomycetes, likewise plating 0.10 ml of soil suspension.

2. Take the glass hockey stick spreader, dip it in ethanol, and flame the spreader in a Bunsen burner just long enough to ignite the ethanol.

3. Moving the spreader out of the flame and holding it just above the first of the inoculated plates allows all of the ethanol to burn off. Then quickly open the plate, holding the lid nearby in one hand. Touch the spreader to the agar away from the inoculum to cool it, and spread the drop of inoculum around on the surface of the agar until all traces of free liquid disappear (the surface will become somewhat tacky).

4. Replace the lid, reflame the spreader, and repeat with the next plate. Work quickly so as not to contaminate the agar with air-borne organisms.

5. Incubate the bacteria plates (inverted) at room temperature for one week.

Making Spread Plates

For Actinomycetes

1. Use the dilutions 10^{-2}, 10^{-3}, and 10^{-4} from above. Spread plate 0.1 ml of vortexed suspension on glycerol-casein plates as you did above, make three replicates for each dilution.

2. Incubate the actinomycete plates (inverted) at room temperature for two weeks.

Third Period

Materials

incubated bacteria plates from Period 2
5 peptone-yeast agar plates or R_2A agar plates
inoculation loop
ethyl alcohol
gas burner
marking pens

Counting Bacteria (after 1 week incubation)

1. Examine all of the bacteria plates carefully. Note differences in colony size and shape.

2. Count the total number of bacterial colonies (CFUs) for each plate, including any actinomycetes. Average the totals for each dilution. Count only those plates of a dilution that are countable (30–200 colonies per plate).

3. Calculate the sample mean of CFUs per gram of dry soil for each of your soils. The calculation is similar to that used in the soil fungi experiment, except that the effective dilution is increased by one order of

magnitude since only 0.1 ml of inoculum was used for the spread plate as opposed to 1 ml in the pour plates. Also, calculate for each soil the sample standard deviation and the coefficient of variation.

Isolation of Pure Cultures

1. Select five individual bacterial colonies from any of the plates. Use a high dilution plate as it will tend to have pure colonies that are well separated. Choose only colonies that are well separated from neighboring colonies and look morphologically distinct from each other. Include an actinomycete as one of the colonies.

2. Sterilize your loop by dipping it in alcohol and flaming it.

3. Quickly open the Petri dish of interest, and touch the loop to a bare spot in the agar to cool it. Then, remove a small amount of a colony of interest onto the loop.

4. Open a fresh peptone-yeast plate and quickly make a streak as shown by streak A in Figure 5-2.

5. Sterilize the loop again, touch a bare spot on the agar on the new plate, and make streak B, crossing streak A only on the first pass. If you cross A again, you will not succeed in isolating individual colonies.

6. Repeat the previous step, making streak C, crossing only streak B on the first pass.

7. Finish with streak D, crossing only streak C on the first pass. If performed properly, this technique will result in individual colonies growing on streak D or sooner, as the number of cells on the loop were sufficiently diluted to individual cells.

Fourth Period

Materials

incubated actinomycete plates from Period 2
incubated isolation plates from Period 3
Gram stain reagents: crystal violet, iodine, decolorizer, and safranin
inoculating loop
ethyl alcohol
5 microscope slides
gas burner
staining rack
pan to catch excess stain and rinseate
sterile water in a capped test tube
2 clean, sterile test tubes
3 sterile 1 ml pipettes
pipette bulb
vortex
2 peptone-yeast agar plates
2 disks treated with each of 3 antibiotics
forceps

Counting Actinomycetes (after 2 week incubation)

1. Examine all of the actinomycete plates carefully. Note differences in colony size and shape.

2. Count the total number of actinomycete colonies (CFUs) for each plate, subtracting any bacteria. Average the totals for each dilution. Count only those plates of a dilution that are countable (as for the bacteria).

3. Calculate the sample mean of actinomycete CFUs per gram of dry soil for each of your soils. Also, calculate the standard deviation and the coefficient of variation.

Gram Stain

1. Examine your bacterial streak plates after one week. Observe the colonies for uniformity of shape and size. Note the presence of any contaminant and indicate in your report whether you feel your isolates are pure.

2. Transfer a small drop of tap water to a slide with a wire inoculating loop. Flame the wire loop and remove a small amount of culture. Mix the bacteria in the drop of water, spreading it over an area about the size of a dime.

3. Let the smear air-dry and then fix the film by passing the slide through the Bunsen burner flame 2 or 3 times.

4. Apply 5 drops of crystal violet to the smear, allowing the dye to remain on the slide for 2 or 3 minutes.

5. Rinse the slide with water and then with iodine solution. Cover with fresh iodine and let stand for two minutes. Rinse with water, using a gentle stream.

6. Decolorize with decolorizer. Add the decolorizer drop by drop to the smear with the slide held tilted. Continue decolorization until no more stain is seen to wash from the smear (usually 20 seconds is sufficient). Rinse immediately in water.

7. Counterstain for 10 seconds with safranin and rinse the slide with water.

8. Carefully blot the slide to hasten drying. Examine the dry preparation under oil using the oil immersion objective. Observe and sketch details.

Antibiotic Resistance

1. Transfer 0.3 ml of sterile water from a fresh tube to each of two clean, microfuge tubes using a sterile 1 ml pipette. Recap the new tube.

2. Select one gram-positive and one Gram-negative bacterium.

3. Take a representative loop and transfer it to a corresponding labeled tube containing 0.3 ml of sterile water. Shake or vortex to disperse.

4. Transfer 0.1 ml from each tube and spread on a fresh peptone-yeast plate. You will have one spread plate for each bacterium (a total of two plates). Label the plates.

5. To one plate of each bacterium add three antibiotic disks (commercially available), each containing a different antibiotic. See Box 1 for the spectra of these antibiotics.

6. Place the disks toward the edge of the plates using flame-sterilized forceps, so that the disks are evenly spaced (see Figure 5-3).

7. Press lightly on the disks so that they will not fall when the plate is inverted.

8. Invert the plates and incubate them at room temperature for one week.

Fifth Period

Materials
incubated antibiotic resistance plates from Period 4
centimeter ruler

1. Examine plates and look for clearing zones around the disks. Measure the radius of the clearing zones from the center of the antibiotic disk for each disk. If the zones overlap, use your best estimate.

2. Discuss the effectiveness of each antibiotic on such bacteria. Keep in mind that antibiotics may or may not be specific to certain types of organisms.

5.4. TRICKS OF THE TRADE

DO:

- Add water to soil without stirring to avoid "puddling" the soil
- Plate from three successive dilutions to ensure getting countable numbers of colonies
- Change pipettes appropriately when plating different dilutions
- Add only 0.1 ml of soil inoculum to each plate, and take this into account in the calculation as an "extra" order of magnitude of dilution
- While preparing streak plates, after sterilizing the loop let the loop cool prior to streaking, or all microbes will be heat killed
- During Gram staining, wear gloves (or you'll be sorry!)

DO NOT:

- Allow soil to settle during preparation of soil dilutions
- Attempt to isolate pure culture colonies from plates that are overcrowded since this will likely result in "mixed" contaminated cultures

- During heat fixing of cells on the microscope slide for the Gram stain, do not "fry" the cells
- Add too much decolonizer during Gram staining, as this will remove all stains and nothing will be seen under the microscope
- During Gram staining do not confuse gram variable *Arthrobacter* with a contaminated "pure culture" isolate. Check your streak plate and examine colony morphology of isolated colonies to ensure that you have pure cultures

5.5. POTENTIAL HAZARDS

- Use gloves when handling cycloheximide
- Keep ethanol away from Bunsen burner at all times
- Use gloves when handling antibiotic discs

5.6. QUESTIONS AND PROBLEMS

1. Note differences in bacterial colony size and shape.

2. Calculate the mean number of total bacterial CFUs per gram of dry soil (bacteria + actinomycetes) for each of your soils. Calculate the standard deviation for each sample mean and the coefficient of variation.

3. Note differences in actinomycete colony size and shape.

4. Calculate the mean number of actinomycete CFUs per gram of dry soil for each of your soils. Calculate the standard deviation for each sample average and the coefficient of variation.

5. Observe the colonies of your streak plates for uniformity of shape and size. Note the presence of any contaminant and indicate in your report whether you feel your isolates are purely based on colony morphology.

6. Describe and sketch details of your Gram-stained slides as viewed under the oil immersion objective of your microscope

7. Report the radius of the clearing zones from the center of the antibiotic disk for each disk.

8. Discuss the effectiveness of each antibiotic on each type of bacteria. Keep in mind that antibiotics may or may not be specific to certain types of organisms.

9. In what respects are actinomycetes closely related to bacteria. What are the similarities with fungi?

10. What are the predominant soil factors that influence bacterial and actinomycete populations in soil?

11. Discuss the effect that the type of medium used in plating can have on your results.

5.7. REFERENCES

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Pepper, I.L., and Gentry, T.J. (2002) Incidence of *Bacillus anthracis* in Soil. *Soil Science* **167**, 627–635.

Pepper, I.L., Gerba, C.P., and Brusseau, M.L. (1996) *Pollution Science*. Academic Press, San Diego.

Pillai, S.D., and Pepper, I.L. (1991) Transposon Tn5 as an identifiable marker in rhizobia: Survival and genetic stability of Tn5 mutant bean rhizobia under temperature stressed conditions in desert soils. *Microbial Ecology* **21**, 21–33.

Algae: Enumeration by MPN

EXPERIMENT 6

6.1. OVERVIEW

Objective: ***To enumerate soil algal populations using a most probable number (MPN) technique.***

- Adjust soil moisture of soil to a value close to field capacity (value provided by instructor)
- Adapt soil to new moisture content by incubating for one week
- Inoculate tubes of algae media with soil dilutions
- Observe tubes for growth after four weeks of incubation
- Calculate soil algae concentrations via a most probable number (MPN) procedure

6.2. THEORY

Algae are eukaryotic phototrophic organisms, requiring light for photosynthesis. Therefore, most algae cells would typically be found at the soil surface. However, some algae can grow heterotrophically and can be found at soil depths of 1 m (Maier et al., 2000). Algae are important colonizers of developing soils that lack organic matter. They can also help in soil aggregation through secretion of extracellular polysaccharides (Killham, 1994).

The term algae refers to a heterogeneous group of organisms that can be motile or nonmotile, and eukaryote or prokaryote. Historically the prokaryotic algae were known as the "blue green algae." Some form of prokaryotic algae is believed to have been the ancestor of the chloroplasts found in eukaryotic algae and plants. These prokaryotic organisms are now classified as cyanobacteria, i.e., as bacteria. The cyanobacteria are also able to fix atmospheric nitrogen, and are sometimes found on the surface of arid soils following rainfall events. Eukaryotic algae are also common under such situations. In fact, when one considers only the surface soil, algal numbers can reach 10,000 organisms per gram of dry soil (Maier et al., 2000). However, beneath the soil surface, algal numbers are several orders of magnitude lower than the bacteria, actinomycetes, or fungi, as algae need light to grow.

As algae are photoautotrophic organisms, the media used to isolate them are made exclusively with essential macro- and micronutrients. As no oxidizable carbon is present, rapidly growing heterotrophic bacteria pose no competitive threat.

Algae can be enumerated and otherwise analyzed in soil by a number of methods including the most probable number procedure (MPN). The

Most Probable Number procedure is a statistical method based on diluting organisms to extinction, and evaluating whether a positive sign of the organism's presence (such as color change in the medium or gas formation) appears in replicate tubes from a given dilution. As the calculations involved are very tedious, tables have been published that are specific to a particular MPN design. MPN is commonly used in soil analyses for such assays as pathogen detection and in wastewater analysis.

The procedure is depicted in Figure 6-1. A dilution series is made much as for the culturable heterotrophic plate counts in the previous experiment. However, 5 tubes of media will each be inoculated with 1.0 ml from each dilution suspension (step 2). The tubes must be incubated with sunlight or artificial growth lights.

The MPN evaluation itself is simple and involves little more than counting the tubes that show a positive sign of growth (generally a green surface ring or film (pellicle)) and using the data in an MPN table along with soil dilution information. See the Calculation section for details.

6.3. PROCEDURE

Materials

25 g fresh soil for each soil type
one plastic container for each soil type
weighing dishes
bench top balance (±0.01 g)
deionized water
plastic wrap
rubber bands
marking pens
dissecting probe

First Period

1. Weigh out a 25 g sample of soil by fresh weight into a labeled plastic container, amending the soil moisture to a value given to you by the instructor.

2. Cover the containers with Saran wrap, secure the wrap with a rubber band, and puncture the wrap two times with a probe to allow air in. Weight the soil and wrap to track soil moisture loss during incubation at room temperature for one week.

Second Period

Materials

incubated soil from Period 1
bench top balance (±0.01 g)
weighing dishes
1 sterile, 95-ml water blank for each soil type
30 9 ml blanks of modified Bristol's solution for each soil type
10 sterile, 1 ml pipettes for each soil type
1 test tube rack with at least 5 openings
1 test tube rack with at least 25 openings for each soil type
pipette bulb
marking pens
dissecting probe
vortex mixer

1. Re-weigh the soil sample with the wrap and rubber band and record the weights to determine the moisture content of the soil after incubation for one week.

2. Prepare a 10-fold dilution series of 10^{-1} to 10^{-6} g soil ml^{-1} using 10 g of the soil, one 95 ml and five 9 ml blanks containing a modified Bristol's solution (see Figure 6-1).

3. For each of the dilutions 10^{-2} to 10^{-6} inoculate five 9 ml blanks of modified Bristol's solution by aseptically transferring, for example, 1 ml of the contents of tube B to each of five blanks, as shown in step 2 in Figure 6-1. Incubate the capped tubes four weeks in a greenhouse, or growth chamber.

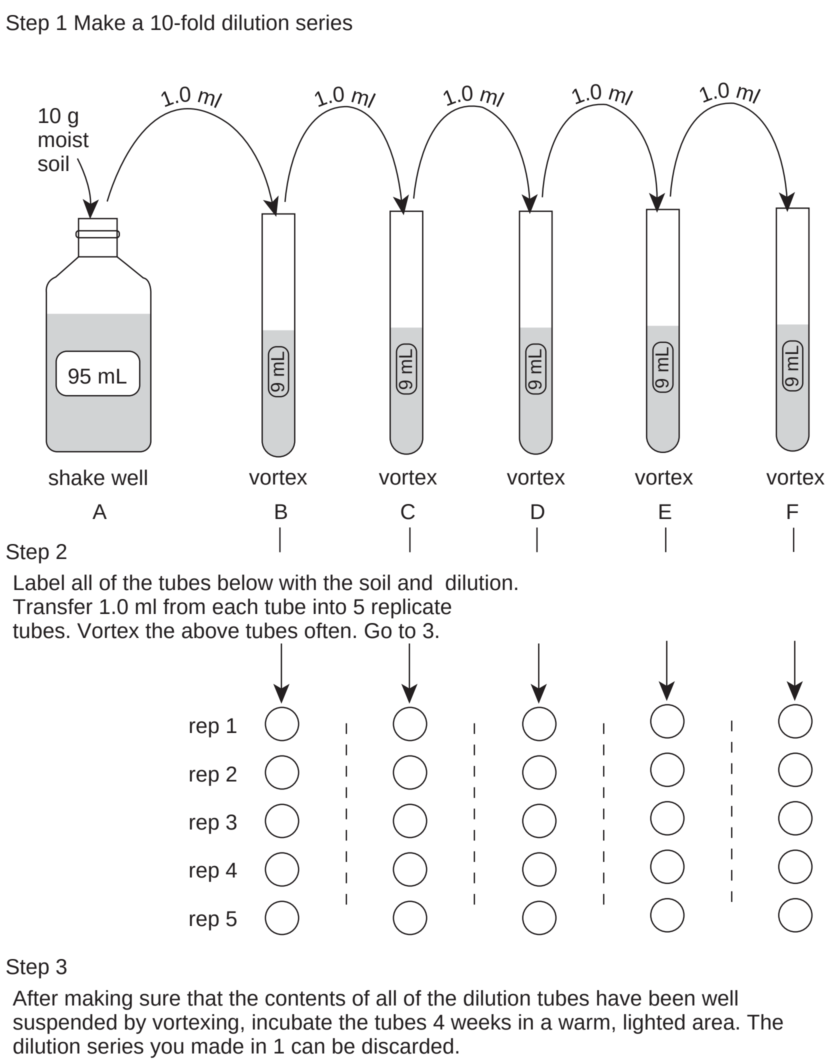


Figure 6-1 Schematic showing the procedure for determining soil algal counts by MPN analysis.

Third Period

Materials
incubated tubes from Period 2

1. Observe your tubes and record the number of tubes showing a positive sign of growth for each dilution. Calculate the number of algae on a dry soil weight basis and the upper and lower 95% confidence limits as described in the Calculation section. Do not forget to take into account any drying which may have occurred during the initial incubation period.

6.4. TRICKS OF THE TRADE

DO:

- Be careful preparing soil dilutions—do not allow soil to settle
- Provide appropriate light—natural light or use a growth chamber

DO NOT:

- Give up on the algae! Allow several weeks of incubation.

6.5. POTENTIAL HAZARDS

- Nothing obvious, but never underestimate student ingenuity!

6.6. CALCULATIONS

Two examples of applying MPN to enumerating algae will be given in the following discussion. The dilution procedure used is shown in Figure 6-1. Assume the actual amounts of dry soil in each tube range from 8.2×10^{-3} g (Tube B) to 8.2×10^{-7} g (Tube F). (Remember 10 g of moist soil were originally weighed out, therefore actual dry weight of soil depends on soil moisture at the time of diluting.)

Example 1

Figure 6-2 depicts the appearance of the tubes after incubation. Shaded tubes indicate positive signs of growth and empty tubes negative signs of growth.

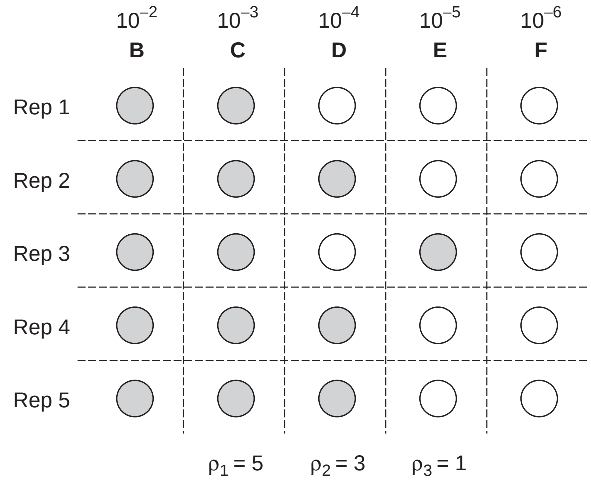


Figure 6-2 Hypothetical outcomes of an algal enumeration assay by MPN discussed under Example 1 in the Calculations section. Shaded circles represent tubes with a positive sign of growth. Empty circles represent tubes with no sign of growth and are therefore negative.

Choose ρ_1 to be the number of replicate tubes of the highest dilution (least concentrated in soil) that has the highest number of positive tubes. Here, the replicates from Tube B do not count because those of Tube C are from a higher dilution. In contrast, the number of tubes from Tube D that show a positive sign of growth is less than those from Tube C. So, $\rho_1 = 5$.

Choose ρ_2 and ρ_3 to be the number of tubes in the next two higher dilutions that show a positive sign of growth. Thus, $\rho_2 = 3$ and $\rho_3 = 1$. Look down the first column in Table 6-1 to find your value for ρ_1. Do the same in the ρ_2 column. Then find where your value of ρ_3 (across the top) intersects the row defined by your values of ρ_1 and ρ_2. In this example, the value is 1.1 organisms ml^{-1}.

Divide the tabular value by the concentration of soil in the dilution to which you assigned Δ_2 (Tube D) to obtain the number of organisms per gram of dry soil:

Therefore, $\frac{1.1 \text{ organisms}}{\text{ml}} \times \frac{\text{ml}}{8.2 \times 10^{-5}} = 1.34 \times 10^4$ algal cells g^{-1} soil

The upper and lower 95% confidence limits are calculated from Table 6-2. In this experiment, you have used a 10-fold serial dilution with five tubes per dilution. Looking in Table 6-2, find the dilution ratio you used (10) in the top row and then find where it intersects 5 tubes per dilution (at 3.30).

The upper confidence limit is $1.34 \times 10^4 \times 3.30 = 4.42 \times 10^4$ algal cells g^{-1} soil.

The lower confidence limit is $\frac{1.34 \times 10^4}{3.30} = 4060$ algal cells g^{-1} soil.

Table 6-1 Most probable numbers for use with the experimental design in this exercise. From Alexander (1982). The bolded values are explained in the Calculation section

		Most probable number for indicated values of ρ_3					
ρ_1	ρ_2	0	1	2	3	4	5
0	0	—	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.1	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.16	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.1	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.25	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.5
4	3	**0.27**	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.65	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.7	0.95	1.2	1.5	1.8
5	3	0.79	**1.1**	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16	—

Example 2

Figure 6-3 shows that $\rho_1 = 4$, $\rho_2 = 3$, and $\rho_3 = 0$. Assume ρ_2 had 8.2×10^{-4} g of soil ml^{-1}. The MPN is therefore 0.27 organisms ml^{-1} or 329 algal cells g^{-1} soil. Note that this value is less than the value in Example 1. This is intuitive since less tubes contained algae, even at relatively low soil dilutions. The upper confidence limit is 1086 and the lower confidence limit is 100 g^{-1} soil.

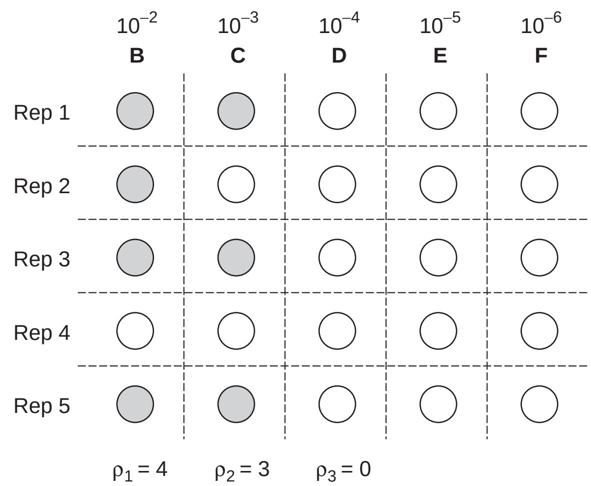


Figure 6-3 Hypothetical outcome of an algal enumeration assay by MPN discussed under Example 2 in the Calculations section. The dilution procedure is described in Figure 5-1. Shaded circles represent tubes with a positive sign of growth. Empty circles represent tubes with no positive sign of growth.

Table 6-2 Factors for calculating the upper and lower 95% confidence intervals for an MPN analysis of the design presented in this laboratory exercise. From Alexander (1982).

Number of Replicate Tubes	**Dilution Ratio**			
ρ_1	**2**	**4**	**5**	**10**
1	4.00	7.14	8.32	14.45
2	2.67	4.00	4.47	6.61
3	2.23	3.10	3.39	4.68
4	2.00	2.68	2.88	3.80
5	1.86	2.41	2.58	3.30
6	1.76	2.23	2.38	2.98
7	1.69	2.10	2.23	2.74
8	1.64	2.00	2.12	2.57
9	1.58	1.92	2.02	2.43
10	1.55	1.86	1.95	2.32

6.7. QUESTIONS AND PROBLEMS

1. Calculate the algal populations in your soil.
2. Calculate the upper and lower 95% confidence limits.
3. How are algal counts related to soil profile depth?
4. Under what conditions would you expect algae to have a competitive advantage over heterotrophic microorganisms in soil?

6.8. REFERENCES

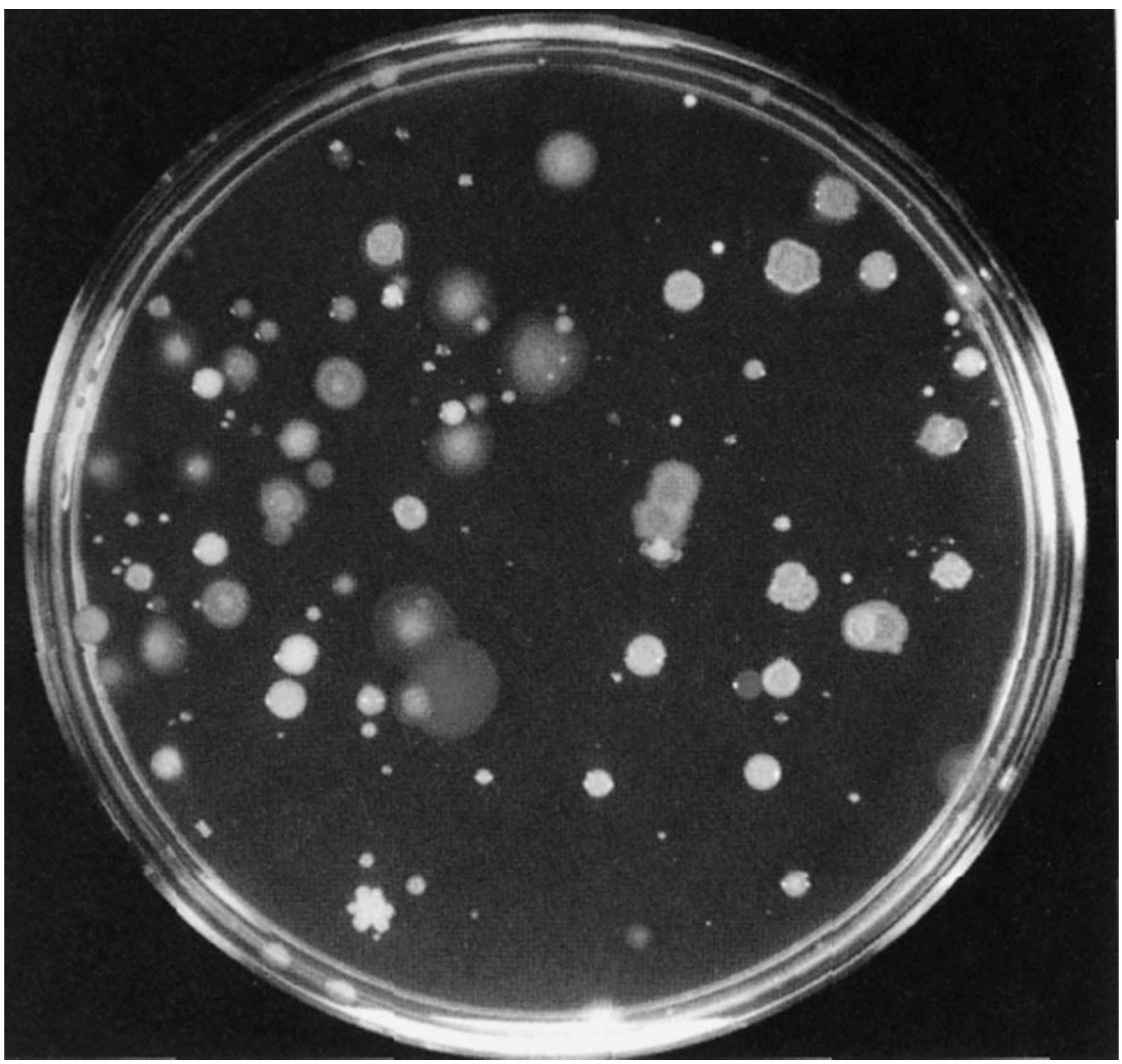
Alexander, M. (1982) Most probable number methods for microbial populations. In: *Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties*, 2nd edition, pp. 815–820. American Society of Agronomy, Inc., Soil Science Society of America, Inc., Madison, Wisconsin.

Killham, K. (1994) Soil Ecology, Cambridge University Press, Cambridge, UK.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

SECTION THREE

Microbial Transformations and Response to Contaminants



Soil bacterial colonies obtained via dilution and plating

Oxidation of Sulfur in Soil

EXPERIMENT 7

7.1. OVERVIEW

Objective: ***To monitor the oxidation of elemental sulfur to sulfate in soil, along with a concomitant decrease in soil pH due to the production of protons.***

- Set up 3 soil incubations: i) unamended control soil; ii) soil and sulfur; and iii) soil and sulfur + glucose
- Adjust soil moisture of all incubations to a value close to field capacity (value provided by instructor)
- Measure initial soil-sulfate concentration and initial soil pH
- Incubate all treatments for 3 weeks
- Evaluate sulfur oxidation in all three treatments by analyzing for soil-sulfate and soil pH at weekly intervals

7.2. THEORY

In addition to enumerations, soil microorganisms can also be analyzed through measurements of physiological activity, where the focus is on enzymes or their activity.

Enzymatic reactions can occur in soil both biotically and abiotically. Thus, in addition to metabolism of substrate by enzymes in living cells, evidence exists that *cell-free* enzymes can also participate in chemical reactions in the soil (Burns, 1982; Skujins, 1976). Some enzymes are known to be active extracellularly, while others are believed to be active only in living cells.

Sulfur oxidation can occur in soil through the activity of chemoautotrophic or heterotrophic organisms, and the outcome of sulfur oxidation can be harmful or beneficial. During strip mining, exposure of previously subsurface sediments that contain sulfides to more aerobic environments can cause sulfur oxidation, a pH decrease, and subsequent mobilization of heavy metals into mining effluents. But in the desert Southwest, fertilizers such as those illustrated in Figure 7-1 routinely contain elemental sulfur in deliberate attempts to cause sulfur oxidation and concomitant pH decreases in alkaline desert soils (Maier et al., 2000). As sulfur oxidizing organisms oxidize elemental sulfur in soil to sulfate, two by-products are formed: SO_4^{2-} and H^+ (acid):

$$S^\circ + \frac{3}{2}O_2 + H_2O \leftrightharpoons H_2SO_4 \quad (7\text{-}1)$$



Figure 7-1 Commercial fertilizers in the Southwest USA contain sulfur to allow soil acidification. (Photo courtesy K.L. Josephson.)

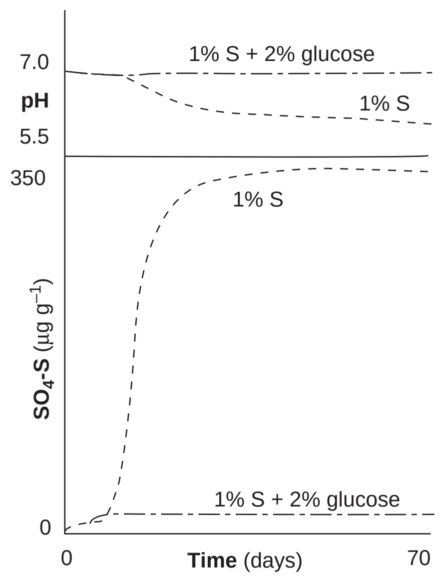


Figure 7-2 Oxidation of sulfur in sterile soil inoculated with *Thiobacillus thiooxidans* with and without a glucose amendment. Note the lack of oxidation in the presence of glucose. (Adapted from Pepper and Miller, 1978.)

Chemoautotrophic organisms, such as *Thiobacillus thiooxidans*, use the energy released by this reaction to fix carbon dioxide. In fact, chemoautotrophic S oxidizers may be inhibited by the presence of easily degraded carbon sources (see Figure 7-2). Thus, soils high in oxidizable organic matter may exhibit low levels of chemoautotrophic sulfur oxidation.

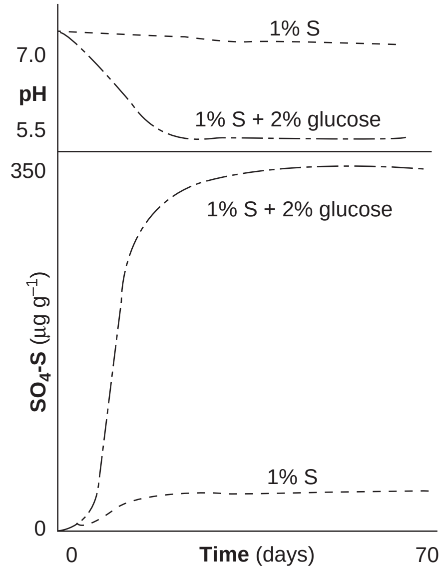


Figure 7-3 Oxidation of sulfur in sterile soil inoculated with a heterotrophic sulfur oxidizing isolate with and without glucose. Note that oxidation only occurs in the presence of glucose. (Adapted from Pepper and Miller, 1978.)

A number of heterotrophic organisms can also oxidize elemental sulfur according to the above reaction (Pepper and Miller, 1978). However, this oxidation occurs cometabolically, and the organisms derive no energy from the reaction, although the products of the reaction are the same with sulfate and H^+ being produced (Figure 7-3). Because these organisms are heterotrophic, no S oxidation occurs unless metabolizable carbon is present. Note that the effect of glucose on S oxidation depends on whether oxidation is by autotrophic or heterotrophic organisms (Figures 7-2 and 7-3).

Yet, another outcome is possible given glucose addition. If other, non-sulfur oxidizing organisms are also stimulated by the easily metabolized substrate, a substantial amount of the produced sulfate may be immobilized in microbial biomass, and the measured soil sulfate concentrations will be reduced (Figure 7-4).

Because sulfate is produced by oxidation of sulfur, the rate of sulfur oxidation can be monitored by measuring changes in the soil sulfate over time. Sulfate is easily extracted from the soil as it is anionic and not retained well on negatively charged soil particles. The use of an NaCl extractant solution is to aid in dispersing the soil particles, exposing their surface to the solution.

After the extracted sulfate is separated from suspension via filtration, the sulfate is precipitated by the addition of $BaCl_2$:

$$BaCl_{2(aq)} + SO^{2-}_{4(aq)} \leftrightharpoons BaSO_{4(s)}\downarrow + 2Cl^{-}_{(aq)} \quad (7\text{-}2)$$

Because $BaSO_4$ is an opaque solid, the solution becomes turbid. Thus, the concentration of sulfate can be measured on a visible light spectrophotometer by turbidimetry. Concentration of sulfate is a linear function of Q, which is a measure of the degree of scattering of light of an optimal wavelength

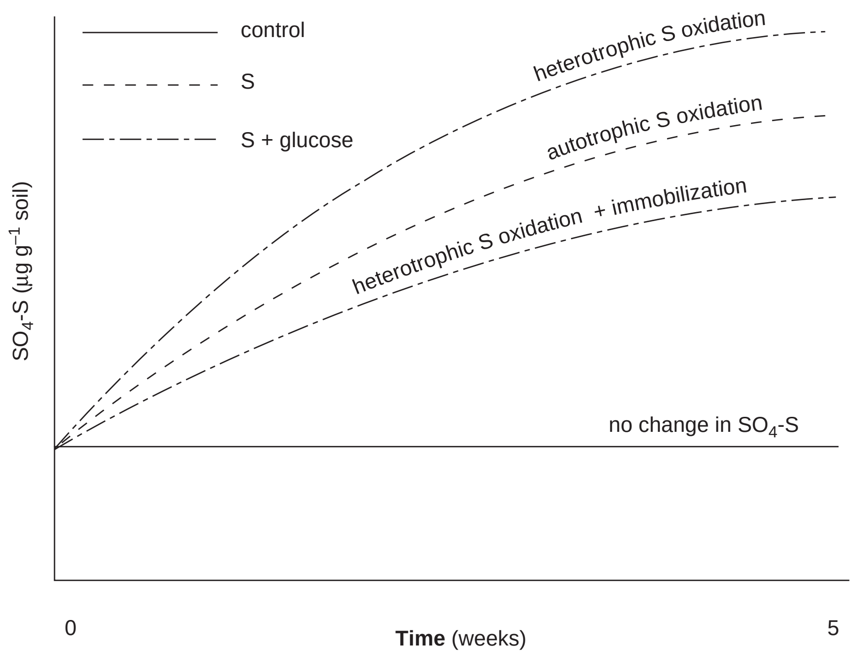


Figure 7-4 Possible effects of S and glucose soil amendments on sulfur oxidation.

Table 7-1 Standard curve data and equation for [SO_4-S] using turbidimetry

X SO_4-S (µg ml^{-1})	*Q*
0.00	0.000
12.5	0.293
25.0	0.478
50.0	0.883
78.0	1.380

Q = absorbance
X = SO_4-S concentration (µg ml^{-1}).

passing (here, 470 nm) through the solution. *Q* is usually read as absorbance on a spectrophotometer. A calibration curve has already been prepared from standard solutions (Table 7-1). Activated carbon is added to the solution to remove any organic compounds which may absorb light at 470 nm, and gum arabic helps keep the $BaSO_4$ particles in suspension.

As acid is also produced during sulfur oxidation, progress of the reaction in soil can also be measured by following changes in the soil pH. In this experiment, you will measure the soil pH over time using a 1:2 suspension of soil in 0.01 *M* $CaCl_2$, a solution commonly used for this purpose.

7.3. PROCEDURE

First Period

Materials
640 g fresh soil for each soil type
3 waterproof containers large enough to hold 250 g of soil for each soil type
analytical balance
benchtop balance (±0.01 g)
weighing dishes
mixing spatula
deionized water
powdered, elemental sulfur
glucose
plastic wrap
rubber bands
pH meter with appropriate reference and H^+ electrodes

pH buffers for calibration (bracket pH range of soil)
wash bottle to rinse pH electrodes
container to catch electrode rinseate
container of tap water to store pH electrodes between measurements
40-ml of 0.01 *M* $CaCl_2$ for each soil type
1 125-ml Erlenmeyer flask (for blank)
2 125-ml Erlenmeyer flasks for each soil type
25 ml 0.1 *M* NaCl for each flask
25 ml graduated cylinder
5 ml graduated pipette
pipette bulb
1 filtration funnel for each flask
Whatman®[1] #42 filter paper
activated charcoal
test tube rack
1 ml of 0.5% (w/v) gum arabic for each flask
$BaCl_2$
spectrophotometer at $\lambda = 470$ nm
at least 2 cuvettes
tissue wipes to clean cuvettes
volumetric flasks and pipettes as needed for dilutions
waste container for barium disposal

1. Weigh out the quantity of soil specified below into large containers and amend (dry weight basis) as follows:
   a) Control-untreated (240 g soil)
   b) 0.5 g elemental sulfur (200 g soil)
   c) 0.5 g elemental sulfur plus 0.5 percent glucose (200 g soil)

2. Mix all amendments uniformly with the soil.

3. Amend the soil with deionized water to the moisture content specified by your instructor. Add the moisture dropwise with a pipette and constant stirring.

4. Cover the containers with plastic wrap and punch holes in the wrap to allow aerobic conditions, securing with a rubber band. Record the weights of the soil + container + wrap + rubber band.

Determination of Initial Activity

At t = 0 weeks, assume that all of the treatments are of the same activity. For this week only, measure soil pH and [SO_4-S] in the control soil.

pH Determination

1. Weigh out duplicate 10 g subsamples of the incubated soils into separate vials. No blank is needed for pH determination.

---

1 Whatman Paper Limited, England.

2 American Can Company, Greenwich, Connecticut.

2. Add 20 ml 0.01 *M* $CaCl_2$, stir, and allow the soil to settle for at least 30 min. Measure the pH to the nearest 0.1 pH unit. Follow the instructor's directions on how to use the pH meter.

[SO_4-S] Determination

1. Weigh out duplicate 10 g subsamples of each soil into 125 ml Erlenmeyer flasks. Use a clean flask as a blank. Add 25 ml of extracting solution (0.1 *M* NaCl). Cover with Parafilm® and shake intermittently for 30 min.

2. Add 0.2 g activated charcoal to each of the flasks and resume shaking for three minutes. This removes any colored organics in the solution.

3. Filter the suspension through Whatman® #42 paper into a 16 × 125 mm test tube, collecting at least 5 ml of the clarified soil extract. It is not important that the volume is exact since we know there were initially 25 ml of extracting solution and we are measuring the amount of sulfate per ml of solution.

4. Using a pipette, transfer 5 ml of the extract to a fresh tube. Add 1 ml of 0.5% (w/v) gum arabic as stabilizer and approximately 0.5 g $BaCl_2$ crystals. This amount is sufficient to precipitate any and all sulfate that was present in the solution. This is an excess of $BaCl_2$ and need not be measured exactly. Cover the tubes with Parafilm® and shake to dissolve the crystals; $BaSO_4$ will precipitate in the tube.

5. Measure the concentration of SO_4^{2-} as turbidity using a spectrophotometer at 470 nm by taking readings of absorbance (*Q*) for each of your extracts. Keep the precipitate suspended in a consistent fashion when measuring the turbidity (gently invert the cuvette prior to measurement while holding Parafilm® on the top). Use the data given in Table 7-1 to prepare a standard curve. From the curve, translate *Q* values into sulfate concentrations ($\mu g\,ml^{-1}$).

6. If the absorbance reading is outside of the calibration curve range ($Q \gg 1.3$), you will need to make the appropriate dilutions of the extract (see the instructor for help). For the purposes of this experiment, use distilled water for your dilutions. See the Calculations section on how to calculate the concentration of sulfate in the soil.

7. Incubate the soils covered at room temperature until the second period.

Second Period (t = 1 week)

Materials

incubated soil samples from Period 1, amended with moisture
benchtop balance (±0.01 g)
weighing dishes
mixing spatula
deionized water

pH meter with appropriate reference and H^+ electrodes
pH buffers for calibration (bracket pH range of soil)
wash bottle to rinse pH electrodes
container to catch electrode rinseate
container of tap water to store pH electrodes between measurements
120 ml of 0.01 *M* $CaCl_2$ for each soil type
1 125-ml Erlenmeyer flask (for blank)
6 125-ml Erlenmeyer flasks for each soil type
25 ml 0.1 *M* NaCl for each flask
25 ml graduated cylinder
5 ml graduated pipette
pipette bulb
1 filtration funnel for each flask
Whatman® #42 filter paper to fit the filtration funnels
1 16 × 125 mm test tube for each flask
Parafilm®
activated charcoal
test tube rack
1 ml of 0.5% (w/v) gum arabic for each flask
$BaCl_2$
spectrophotometer at $\lambda = 470$ nm
at least 2 cuvettes
volumetric flasks and pipettes as needed for dilutions
tissue wipes to clean cuvettes
waste container for barium disposal

1. Weigh the containers with soil and bring them back up to the original weight by adding distilled water. Mix thoroughly afterwards. This also keeps treatments aerobic.

2. Analyze the soils for pH and SO_4-S as described for the first period using soil from the control, S, and S + glucose amendments.

Third Period (t = 2 weeks)

Materials
Same as in Second Period

1. Weigh the containers with soil and bring them back up to the original weight by adding distilled water.

2. Analyze the soils for pH and SO_4-S as described for the first period using soil from the control, S, and S + glucose amendments. If excessive amounts of sulfate are present in the samples, it may be necessary to dilute the sample with distilled water.

7.4. TRICKS OF THE TRADE

DO:
- Use time efficiently, remember that the soil extraction for sulfate requires 30 minutes of intermittent shaking

- Keep incubations aerobic to allow sulfur oxidation to occur
- Remember to reweigh all soil incubations after each weekly analysis to track soil moisture loss
- Adjust soil moisture each week, back to the desired moisture content

DO NOT:
- Contaminate clear soil filtrates with soil via "colloidal creep." This occurs when too much soil suspension is added to the filter, and somehow it ends up in the extract or filtrate!
- Expect to see much of a soil pH decrease if the soil contains free calcium carbonate, as in the case of many desert soils.

7.5. POTENTIAL HAZARDS

DO:
- Dispose of all barium waste in a designated container

DO NOT:
- Expose bare skin to barium solutions

7.6. CALCULATIONS

1. To calculate the [SO_4-S] in the extractants, use the standard curve.

2. To calculate [SO_4-S] per gram dry soil:

$$\frac{\mu g\, SO_4\text{-}S}{g\, dry\, soil} = \frac{\mu g\, SO_4\text{-}S}{ml} \times \frac{25\, ml\, extractant + soil\ moisture}{weight\ of\ dry\ soil\ extracted} \qquad (7\text{-}3)$$

where "soil moisture" refers to the volume of water initially in the 10 g sample of soil you extracted. Note that you do not need to compensate for the 1 ml of gum arabic you added since you treated all soils the same and the standard curve was made in the same fashion.

7.7. QUESTIONS AND PROBLEMS

1. Report in tabular form the concentration of SO_4-S in your soils for each treatment and soil as a function of time. Make a graph showing [SO_4-S] as a function of time. Plot all three treatments (control, elemental S, elemental S + glucose) on one graph, with a separate graph for each soil type.

2. Report in tabular form the pH of your soils for each treatment and for each of the measured times.

3. Discuss the impact of autotrophic and heterotrophic sulfur oxidation on the absolute sulfate concentrations. Similarly, discuss potential immobilization of sulfate.

4. Would you conclude that there is an abundance of sulfate in normal soils?

5. What would be the influence of added organic matter on the oxidation of sulfur in soil?

6. Write an equation that describes the oxidation of elemental sulfur by *T. thiooxidans*.

7. Discuss the major similarities and differences between the microbial transformation of sulfur and nitrogen.

8. Why do fertilizers for use in the southwest USA often contain elemental sulfur?

7.8. REFERENCES

Burns, R.G. (1982) Enzyme activity in soil: Location and possible role in microbial ecology. *Soil Biology and Biochemistry* **14**, 423–427.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Pepper, I.L., and Miller, R.H. (1978) Comparison of the oxidation of thiosulfate and elemental sulfur by two heterotrophic bacteria and *Thiobacillus thiooxidans*. *Soil Science* **126**, 9–14.

Skujins, J. (1976) Extracelluar enzymes in soil. *CRC Critical Reviews in Microbiology* **4**, 383–421.

Dehydrogenase Activity of Soils

EXPERIMENT 8

8.1. OVERVIEW

Objective: ***To demonstrate the dehydrogenase activity associated with soil microorganisms.***

- Set up two soil incubations: i) unamended control soil; and ii) soil + glucose. Also set up a blank—no soil
- Incubate all vials with triphenyltetrazolium chloride (TTC) under saturated soil conditions for 1 week
- Extract both soil treatments and the blank for reddish colored triphenyl formazan (TPF) through the use of methanol
- Analyze TPF concentrations spectroscopically to give an estimate of dehydrogenase activity

8.2. THEORY

Dehydrogenase are enzymes that are found in all living organisms (Maier et al., 2000). These enzymes take part in many reactions involving the transfer of pairs of electrons. An example of a dehydrogenase mediated electron transfer is given in Figure 8-1. In catabolic reactions, i.e., reactions involving the modification of complex or high-energy compounds to simpler or low-energy compounds, dehydrogenases catalyze the transfer of electron pairs from some substrate to NAD^+ forming NADH. NADH then transfers the electrons to another compound, thereby serving as an electron transfer intermediary. In anabolic reactions (the opposite of catabolic reactions), $NADP^+$ is involved instead. Two hydrogen atoms "tag" along to keep the charge balanced; it is the electrons that are being transferred. As dehydrogenases also take part in the electron transfer system of aerobic organisms, the activity of these enzymes is a measure of respiration along with general metabolic activity.

By far the most commonly used version of the dehydrogenase assay is that of Casida et al. (1977). This assay usually involves incubating soil mixed with a solution of the competitive NAD^+ inhibitor, 2,3,5-triphenyltetrazolium chloride (TTC) with soil in air-tight containers to exclude oxygen. Here the TTC serves as the ultimate electron acceptor during respiration. The soil should be freshly collected as research has shown that dehydrogenase assay results are adversely affected by storage, even at 4°C (Ross, 1970). Exclusion of oxygen favors the transfer of the electrons to TTC, reducing the pale yellow, slightly water-soluble compound to the insoluble red dye triphenyl formazan (TPF) (see Figure 8-1).

In fertile soils high in soil organic matter (Figure 8-2), no nutrient amendment is needed, and in fact, one of the advantages of the dehydrogenase assay over other enzyme assays is that it does not require any amendments,

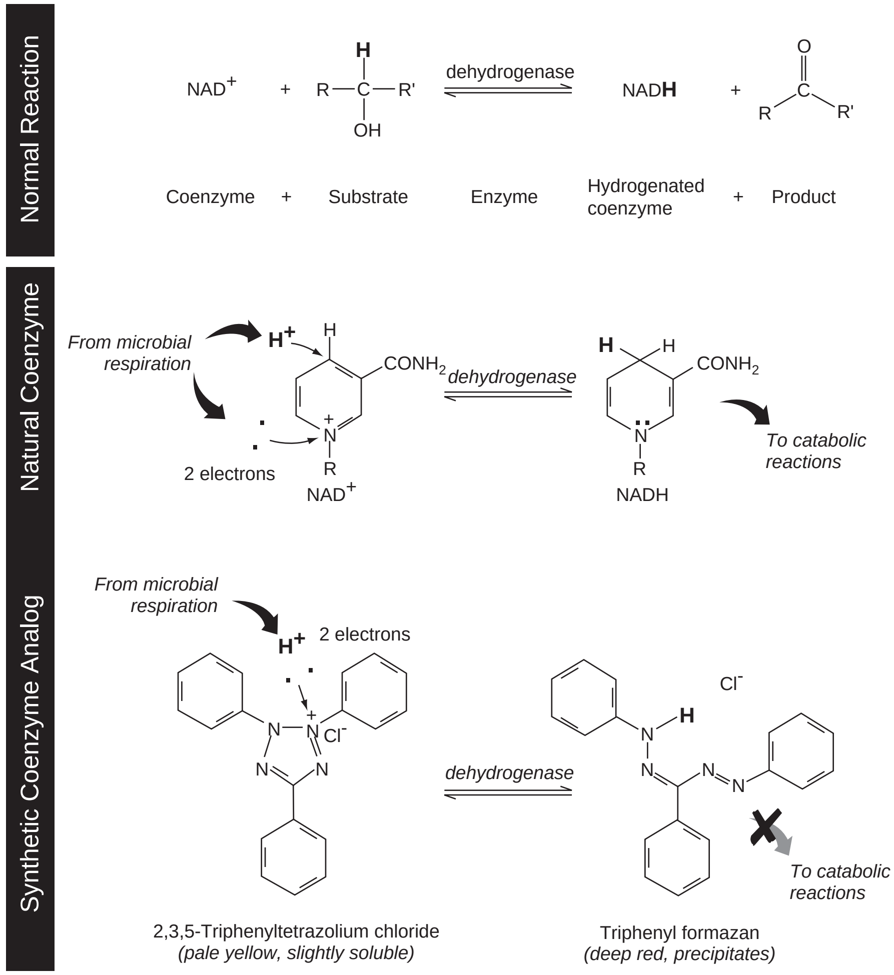


Figure 8-1 **Normal Reaction** NAD^+ is reduced to NADH through a transfer of two electrons and two hydrogen ions in the presence of dehydrogenase from numerous compounds (only one of the two hydrogen ions actually participate in the reaction and, therefore, only one is depicted here).

Natural Coenzyme Here, the chemical transformations occurring to NAD^+ during its reduction to NADH are depicted. The incoming H^+ (in extra bold face type) is reduced in the presence of the dehydrogenase enzyme. The NADH is used to reduce other compounds.

Synthetic Coenzyme Analog TTC 2,3,5-Triphenyltetrazolium chloride (TTC) accepts one H^+ and two electrons in a manner similar to NAD^+. Hence, TTC is being reduced to triphenyl formazan in the presence of dehydrogenase, acts as a competitive NAD^+ inhibitor. However, unlike NADH, the triphenyl formazan is a metabolic dead end. This poisons the system involved but does make it possible to measure metabolic activity due to the red color.

and does not preferentially stimulate any group of microorganisms. However, addition of an organic amendment is necessary in soils of low fertility status, such as desert soils (Klein et al., 1971).

After incubation, the soils are extracted with a solvent to remove the TPF. Casida et al. (1977) used ethanol, which has become the standard. Following extraction, the concentration of TPF in the soil is determined by absorption spectrophotometry. The dissolved analyte, TPF, absorbs light in the visible region of the spectrum, with $\lambda = 485\,nm$ best for analytical purposes. Over a certain range of concentration, the degree to which the TPF absorbs 485 nm light is a linear function of concentration of TPF in the methanol solution.



Figure 8-2 Soils high in soil organic matter have high dehydrogenase activity. (Photo courtesy K.L. Josephson.)

Table 8-1 Standard curve data and equation for (TPF) using visible absorption spectrophotometry at λ = 485 nm. A is the absorbance value. X = the concentration of TPF analyzed in the solution analyzed in the spectrophotometer in $\mu g\,mL^{-1}$

X ([TPF] in $\mu g\,ml^{-1}$)	A
0.00	0.000
5.00	0.214
10.0	0.423
15.0	0.642
20.0	0.833
25.0	1.051
30.0	1.244

$$X = \frac{A - 0.00629}{0.0415}$$

$r^2 = 1.000$

Thus, a standard curve can be developed from absorbance readings of standard solutions. Data for preparation of a standard curve are given in Table 8-1.

8.3. PROCEDURE

First Period

Materials

24 g dry-weight equivalents of fresh soil for each soil type
1 plastic vial with a tightly fitting lid (for blank)

4 plastic vials with tightly fitting lids for each soil type
analytical balance
benchtop balance (±0.01 g)
weighing dishes
mixing spatula
deionized water
1 ml 3% (w/v) TTC for each plastic vial
1 ml pipette
pipette bulb

1. For each soil, weigh 6 g (dry weight) in each of 4 plastic vials. To two duplicate vials, add 0.5% glucose (dry weight basis). Two samples will receive no amendment. Make a blank by adding no soil. This will result in 4 vials with soil for each soil type, and 1 blank.

2. Add 1 ml of 3% TTC and 2.5 ml deionized water to each of the vials, including the blank. Mix the soil and liquid with a glass stirring rod, then put caps on the vials (the vials need to be sealed against oxygen from the air). A small amount of free liquid should just appear at the soil surface. Incubate the vials for one week.

Second Period

Materials
incubated soils from Period 1
methanol
fume hood
10 ml graduated cylinder
1 filtration funnel for each plastic vial
Whatman® #42 filter paper
stand for holding the funnels
50 ml volumetric flasks
5 ml pipette
pipette bulb
cuvettes
spectrophotometer at $\lambda = 485$ nm
tissues for wiping cuvettes
container for collection methanol and TTC wastes

1. To each vial, add 10 ml of methanol, stir, and quantitatively transfer the suspension to a funnel fitted with Whatman® #42 filter paper. Collect the filtrate in a 50 ml Erlenmeyer flask.

2. Wash the vial and the funnel containing the sediment with two more 10 ml methanol rinses until the filtrate is clear of red color. Add more methanol to bring the total volume to 50 ml.

3. Standard curve data is given in Table 8-1. These values are reproducible, so it is not necessary to standardize each time the assay is performed. Should your absorbance readings be outside of the calibration range in Table 8-1, dilute a sub-aliquot of the extract with methanol in a volumetric flask.

4. Transfer 5 ml of each sample to a cuvette. Read the absorbance of each sample on a spectrophotometer at 485 nm using the blank as the zero. Rinse the cuvettes with 1–2 ml rinses of methanol between samples.

5. Express the results as g TPF g^{-1} dry soil. Report the average of the two duplicates.

6. Display the data in a table showing the values for each soil by amendment.

8.4. TRICKS OF THE TRADE

DO:

- Make sure all caps on the vials are tightly sealed to preclude oxygen entry

DO NOT:

- Contaminate the methanol extracts with soil during filtration. This will affect spectroscopic readings.

8.5. POTENTIAL HAZARDS

- Avoid absorption of methanol through skin
- Avoid ingestion of methanol through the mouth!
- Avoid skin contact with TTC

8.6. EXAMPLE CALCULATIONS

Soil Y	Absorbance Value
Container A	0.227
Container B	0.271
Average	0.249

Using the equation in Table 8.1

$$x = \frac{\text{A} - 0.00629}{0.0415}$$

$$\therefore x = \frac{0.249 - 0.00629}{0.0415}$$

$$\therefore x = 5.85\,\mu\text{g/ml of extractant}$$

$$x = \frac{5.85 \times 50}{6}\,\text{g}^{-1}\,\text{soil}$$

$$x = 48.7\,\mu\text{g TPF g}^{-1}\,\text{soil}$$

8.7. QUESTIONS AND PROBLEMS

1. Report the dehydrogenase activity of each soil for the amended and non-amended treatments of each soil.

2. What is a dehydrogenase enzyme?

3. How did glucose influence the results? What would be the result of using another amendment, e.g., powdered wheat straw instead of glucose? What functions do glucose and wheat straw serve in this question?

4. Can microbial enzymes exist in soil outside of microorganisms?

5. How would you interpret your results in light of the fact that you added an amendment to the soil? Is the glucose amendment useful for determining the *in situ* dehydrogenase activity?

6. What are some of the errors that could have occurred in this analysis?

8.8. REFERENCES

Casida, L.E., Jr. (1977) Microbial metabolic activity in soil as measured by dehydrogenase determinations. *Applied and Environmental Microbiology* **34**, 630–636.

Klein, D.A., Loh, T.C., and Goulding, R.L. (1971) A rapid procedure to evaluate the dehydrogenase activity of soils low in organic matter. *Soil Biology and Biochemistry* **3**, 385–387.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Ross, D.J. (1970) Effects of storage on dehydrogenase activities of soils. *Soil Biology and Biochemistry* **2**, 55–61.

Nitrification and Denitrification

EXPERIMENT 9

9.1. OVERVIEW

Objective: ***To monitor important microbial transformations of inorganic nitrogen compounds in soil.***

- Add nitrate to soil and measure semi-quantitatively after one week incubation
- Add an ammonium salt to soil and incubate for one week with the following treatments: i) aerobic; ii) aerobic + glucose; iii) anaerobic; iv) anaerobic + glucose
- Re-measure soil for nitrate to determine whether nitrification and/or denitrification have occurred

9.2. THEORY

Nitrate is a negatively charged ion, which is not absorbed to cation exchange sites which are also negatively charged. In contrast the positively charged ammonium ion is retained. Nitrogen in the form of nitrate leads to reduced plant uptake of nitrogen and lower nitrogen use efficiency. It can also result in contamination of groundwater. Intake of potable water high in nitrates can result in methemoglobinemia. This is also known as "blue baby syndrome" since asphyxiation of infants can occur (Maier et al., 2000). However, nitrification can be a beneficial reaction during wastewater treatment since it allows for subsequent denitrification, thereby reducing the nitrate content of effluent.

Nitrification and denitrification are both essential transformations within the nitrogen cycle. Nitrification involves the oxidation of a reduced form of nitrogen, commonly ammonium, to nitrate in a two-step reaction. Typical nitrifying organisms for the first step belong to the genus *Nitrosomonas*, which convert ammonium to nitrite:

$$NH_4 + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O + \mathit{energy} \qquad (9\text{-}1)$$

The second step in nitrification concerns the oxidation of nitrite to nitrate, a reaction commonly carried out by *Nitrobacter* sp.:

$$NO_2^- + \frac{3}{2}O_2 \rightarrow NO_3^- + \mathit{energy} \qquad (9\text{-}2)$$

Both *Nitrosomonas* and *Nitrobacter* are chemoautotrophic organisms which use the energy derived from the oxidation of ammonium or nitrite to fix

carbon dioxide. Because so much less energy is released from the second step than the first (ca. 100 moles of nitrite must be reduced to fix the same amount of CO_2 as 35 moles in the first step), very little nitrite accumulates in the soil (Atlas and Bartha, 1987).

Control of the nitrification is important in agricultural systems as the negatively charged nitrate is not retained by positively charged soil particles. Nitrification can also occur by a number of heterotrophic organisms (Atlas and Bartha, 1987).

Two forms of denitrification exist: assimilatory and dissimilatory nitrate reduction. Assimilatory nitrate reduction is simply the internal reduction of nitrate to ammonium by a number of organisms, which then convert the ammonium into a wide variety of compounds such as proteins and nucleic acids.

On the other hand, dissimilatory nitrate reduction occurs under exclusively anaerobic conditions by a few, specialized organisms. Normally, denitrifiers are facultative anaerobes which prefer to use oxygen as the terminal electron acceptor in respiration if it is available. However, when oxygen becomes limiting, they can use nitrate as the terminal electron acceptor. Dinitrogen gas is one of the common end products of denitrification. However, the greenhouse gas N_2O is also produced. This contributes to global warming (Pepper et al., 1996). N_2O is easier to measure in analyses of denitrification using gas chromatography than is N_2, as N_2O is naturally present in small concentrations in the atmosphere while N_2 is the largest component in atmospheric gas.

One major heterotrophic organism involved in denitrification is *Pseudomonas denitrificans*:

$$C_6H_{12}O_6 + 4NO_3^- \rightarrow 6CO_2 + 6H_2O + 2N_2 + \textit{energy} \qquad (9\text{-}3)$$

Here, the degree to which denitrification takes place is limited by the amount of oxidizable carbon present in the system.

An important autotrophic organism is *Thiobacillus denitrificans*:

$$2NO_3^- + S + H_2O + CaCO_3 \rightarrow CaSO_4 + N_2 + \textit{energy} \qquad (9\text{-}4)$$

The methods for quantitative measurements of nitrification and denitrification are fairly time and equipment intensive. Here, a semiquantitative test for measuring nitrate in the soil is used utilizing commercial nitrate test strips (EM Quant[1]).

The nitrate test strips show changes in the concentration of solution nitrate as gradation of color on the treated sensing pad on the strip. The sensing pad is then matched up with a color scale printed on the container to determine the concentration of nitrate in terms of ppm ($\mu g\,ml^{-1}$).

An illustration of the strips is shown in Figure 9-1. The nitrate strips have two pads. Actually, both pads change color in response to nitrite. However, only the bottom pad has a reagent that can reduce nitrate to nitrite causing the

1 EM Science, Division of EM Industries, Inc., Gibbstown, New Jersey.

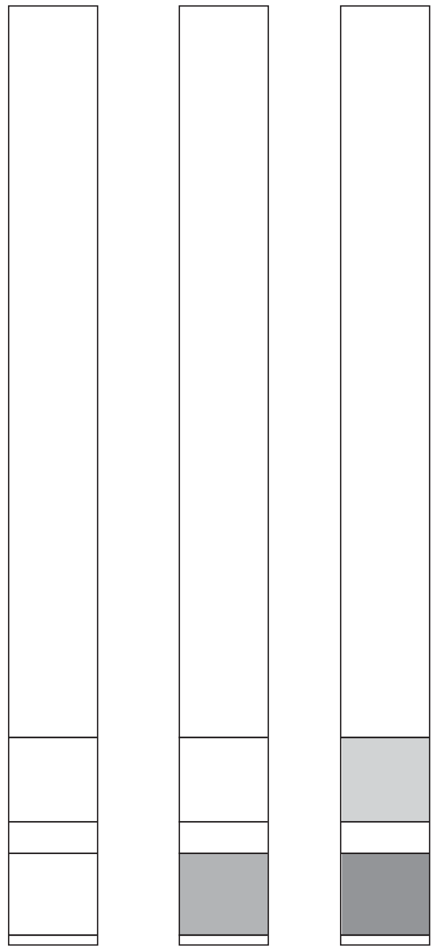


Figure 9-1 Diagram of the nitrate test sticks you will be using. Darker gray on the lower patch denotes higher nitrate concentration while discoloration on the upper patch indicates the presence of the nitrite interferant. The strip on the left shows no nitrate in the solution as no color change occurred. The center strip shows a fairly large amount of nitrate present, but no nitrite. The strip on the right, in contrast, shows amounts of nitrate in the solution that exceed the calibration, and thus, this solution mus be diluted to allow for an accurate analysis. Additionally, significant amounts of nitrite are also present. The nitrate strips have also been known to produce inaccurate results when used with soils that contain interfering concentrations of certain cations, including calcium.

color change to occur. Consequently, the upper pad serves as an indicator of the quantity of nitrite interferant.

9.3. PROCEDURE

First Period

Materials

400 g fresh soil for each soil type
4 containers large enough to hold 100 g of fresh soil for each soil
2 plastic vials for each soil type
KNO_3

analytical balance
benchtop balance (±0.01 g)
weighing dishes
mixing spatula
deionized water
dissecting probe
plastic wrap
rubber bands

1. Add 4 × 100 g (moist weight) of soil into 4 containers. Amend all of the soils with 0.10% KNO_3 (dry weight basis). Thoroughly mix the soil with a spatula.

2. Weigh out 2 × 25 g soil into 2 containers without nitrate.

3. Amend soil moisture to a value prescribed by your instructor. Cover the container with plastic wrap and punch holes. Record the weight of the soil + container + wrap + rubber band. Incubate the soils for one week at room temperature in your drawer.

Second Period

Materials
incubated soils from the previous week
benchtop balance (±0.01 g)
weighing paper and dishes
6 125-ml Erlenmeyer flasks for each soil type
deionized water
25-ml graduated cylinder
1 plastic vial for each soil type
1 filtration funnel for each flask
Whatman®[1] #42 filter paper
stand for holding funnels
1 nitrate test strip for each flask
volumetric flasks and pipettes as needed for dilution
pipette bulb
$(NH_4)_2SO_4$
glucose
mixing spatula

Nitrate Concentration at One Week

1. Weigh all soils with containers and record the weights.

2. Calculate the new moisture content.

3. Weigh out a 10 g sample of soil into each of six 125 ml Erlenmeyer flasks (4 nitrate amended and 2 control soils without nitrate). Record the weight of the soil left in the containers (including covering and rubber bands). Use one empty flask (no soil) as a blank (7 flasks total).

4. Add 25 ml of deionized water to each flask and swirl intermittently for 30 min.

5. Filter the suspension through Whatman® #42 paper into a clean, labeled plastic vial. Only a few milliliters of filtrate are needed.

6. Dip a nitrate test strip into the solution, using the color quantification scale to estimate the concentration of nitrate in the solution (follow the instructions for using the test strips). This gives readings for NO_3^- at t = one week. You may need to dilute the solution to bring the nitrate level to within that of the calibration scale.

7. Calculate the amount of nitrate present ($\mu g\,g^{-1}$ soil) for each sample you filtered. Note whether NO_2^- was present.

Add Ammonium to the Soil

1. Amend the 4 soil samples (earlier amended with nitrate) with 0.1% $(NH_4)_2SO_4$ on a dry weight basis, recording the exact amount that you added. Mix thoroughly with a spatula.

2. Label each of the containers with one of the following: "aerobic," "aerobic + glucose," "anaerobic," "anaerobic + glucose."

3. Add glucose to each of the two glucose treatments on a 0.5% dry weight basis, mixing thoroughly with a spatula.

4. Bring the soils labeled "aerobic" or "aerobic + glucose" back up to their original moisture content (as it was at the beginning of the first period). Cover these soils with the original coverings and re-weigh.

5. To the treatments labeled "anaerobic" and "anaerobic + glucose," add enough distilled water to saturate the soils. Add the water slowly with frequent stirring.

6. Replace the original coverings, weigh, and to the anaerobic treatments only, add a second covering, this time *not* punching any holes. This will restrict air movement into the paste, encouraging anaerobic conditions. Weigh containers.

Third Period

Materials
incubated soils from the previous week
benchtop balance (±0.01 g)
weighing paper and dishes
5 125-ml Erlenmeyer flasks for each soil type
deionized water
25-ml graduated cylinder
1 plastic vial for each soil type
1 filtration funnel for each flask
Whatman® #42 filter paper
stand for holding funnels
1 nitrate test strip for each flask

volumetric flasks and pipettes as needed for dilution
pipette bulb
mixing spatula

Take the Final Readings

1. Weigh each container and record the weights. Calculate the new soil moisture contents.

2. Analyze each of the soils for nitrate using 10 g soil samples as you did in the second period. Calculate the amount of nitrate left in the soils.

9.4. TRICKS OF THE TRADE

DO:

- Keep aerobic treatments aerobic by allowing access to oxygen via holes in the plastic rap

DO NOT:

- Allow anaerobic treatments to be exposed to oxygen

9.5. POTENTIAL HAZARDS

- None

9.6. ASSIGNMENT AND QUESTIONS

1. Report in tabular form the concentration of nitrate in your soils as a function of time.

2. Make a graph of the data showing nitrate concentration in the soils as a function of time, putting all of the amendments on one graph (use different lines and/or symbols for each).

3. What was the purpose of dividing the soils into aerobic and anaerobic treatments? How do these treatments affect nitrification and denitrification processes? Include in your answer the generic names of the organisms involved in these processes.

4. What was the purpose of adding glucose to each of the treatments?

5. What was the fate of the NH_4-N added in each of the treatments?

6. What affected the amount of nitrate left in the following: a) aerobic and aerobic + glucose treatments, b) anaerobic and anaerobic + glucose treatments?

7. Identify one beneficial and one adverse effect of nitrification.

8. Give an example of an important autotrophic denitrifying bacterium.

9.7. REFERENCES

Atlas, R.M., and Bartha, R. (1987) *Microbial Ecology: Fundamentals and Applications*, 2nd edition. The Benjamin/Cummings Publishing Company, Inc., Menlo Park.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press, San Diego.

Pepper, I.L., Gerba, C.P., and Brusseau, M.L. (1996) *Pollution Science*. Academic Press, San Diego.

EXPERIMENT 10

Enrichment and Isolation of Bacteria that Degrade 2,4-Dichlorophenoxyacetic Acid

10.1. OVERVIEW

Objective: ***This experiment will illustrate the enrichment for bacteria that can degrade 2,4-dichlorophenoxyacetic acid (2,4-D), following soil amendment with 2,4-D.***

- Amend soil with 2,4-D
- Incubate soil microcosms for 3 weeks
- Monitor 2,4-D degradation via 2,4-D screening broth
- Dilute and plate to enumerate 2,4-D degraders after 0, 1, and 3 weeks

10.2. THEORY AND SIGNIFICANCE

This procedure, also referred to as selective or elective culture is based on the Darwinian concept of natural selection. This concept states that the organism best able to exploit a particular niche (specific substrate utilization) under all other environmental constraints (temperature, pH, O_2) will be the one that is selected. In soil enrichments, it is not the intent to simulate natural soil nutrient conditions, since these are suboptimal for microbial growth. Rather, the soil is amended with a particular substrate to provide optimal conditions for the rapid isolation of an organism with a particular catabolic phenotype. One method is to repeatedly amend a soil with a particular substrate over an extended period of several weeks. The addition of the substrate will provide a competitive edge to those organisms capable of its metabolism. Consequently, numbers of those organisms will proliferate increasing the likelihood of their isolation during subsequent dilution and plating. Generally, bacteria predominate over fungi in enrichment cultures, perhaps due to the greater metabolic diversity of bacteria, and also the ability to rapidly proliferate via binary fission. In this experiment, we will isolate soil bacteria capable of degrading the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). This will be accomplished by enriching a soil with 2,4-D and incubating the soil for several weeks, followed by dilution and plating on a selective, differential enrichment medium called eosin-methylene blue (EMB)—2,4-D agar (Neilson et al., 1994). This agar contains minimal salts and 2,4-D as the sole carbon source. Indicators eosin B and methylene blue allow for the selection of gram-negative bacteria and differentiation of 2,4-D degrading colonies, which turn black. In essence, this is using 2,4-D as an elective carbon source, and the indicators as both selective agents and differential agents that distinguish on the basis of the black to purplish colonies that arise during 2,4-D degradation. Most soils contain indigenous 2,4-D

degraders, but numbers dramatically increase following exposure to 2,4-D (Newby et al., 2000).

10.3. PROCEDURE

First Period

Materials
2 × 150 g soil
microcosm jars (600 ml polypropylene)
2,4-D indicator plates (EMB 2,4-D agar)
9 ml dilution water blanks
95 ml dilution water blank
1 ml pipettes
glass hockey stick spreader
ethyl alcohol for flame sterilization
vortex mixer
gas burner
benchtop balance (±0.01 g)
spatula
1% 2,4-D stock solution

1. Weigh out 150 g of soil into each of two microcosm jars. Label one as control and one as 2,4-D enrichment.

2. For the control soil, calculate the amount of water that needs to be added to the soil samples to bring it up to the moisture specified by your instructor. This soil moisture content is often close to field capacity. Mix the soil.

3. For the 2,4-D enrichment add the same total volume of water to the soil as calculated for the control soil, but include in this volume enough 1% 2,4-D stock solution to obtain a final soil concentration of 500 μg 2,4-D g^{-1} dry soil. Mix to obtain a uniform 2,4-D concentration throughout the soil.

4. Put the lids on the microcosm jars but do not screw on tightly. This will allow aeration and yet preclude moisture loss. Weigh the 2,4-D enriched microcosm jars and incubate at 25°C or room temperature for one week.

5. Prepare a dilution series for the unamended soil for plating on 2,4-D indicator plates. Specifically use 10 g of soil and dilute in a 95 ml water blank to obtain a 10^{-1} dilution. Re-weigh the control jar after removing the soil to get a final weight. Incubate at 25°C or room temperature for one week.

6. Subsequently transfer a 1 ml aliquot to a 9 ml dilution blank to obtain a 10^{-2} dilution.

7. Spread plate 0.1 ml of each dilution onto 2,4-D indicator plates to give final dilutions of 10^{-2} and 10^{-3} respectively. Use duplicate plates for each dilution.

8. Incubate plates at 25°C for one week. These plates will give an estimate of the original population of bacteria in the soil capable of degrading 2,4-D. Normally these numbers will be low.

Second Period

Materials

2,4-D plates from first period
soil microcosms from the first period
reagent grade 2,4-D
spatula
2,4-D indicator plates (EMB 2,4-D agar)
2,4-D screening broth
2 sterile screw cap tubes each containing 5 ml of 2,4-D screening broth
9 ml dilution water blanks
95 ml dilution water blank
1 ml pipettes
glass hockey stick spreader
ethyl alcohol for flame sterilization
vortex mixer
gas burner

1. Re-weigh microcosms and add H_2O to bring soil moisture content back to original value. Be sure to include the weight of the lid in the total weight as you did in the first period.

2. Remove a 0.5 g sample of soil from each microcosm and add individually to a 5 ml screening broth tube. This screening broth contains 2,4-D and an indicator dye. Place each tube on a shaker and observe growth over the next 2 to 3 weeks. A change in color from green to yellow indicates 2,4-D degradation is occurring, and that degrading organisms are present.

3. Prepare a dilution series for each soil for plating on 2,4-D indicator plates. Specifically use 10 g of soil and dilute in a 95 ml water blank to obtain a 10^{-1} dilution.

4. Subsequently transfer 1 ml aliquots to 9 ml dilution blanks to obtain dilutions through 10^{-3}.

5. Spread plate 0.1 ml aliquots of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions on 2,4-D indicator plates. Note that your final dilutions will be 10^{-2}, 10^{-3}, and 10^{-4} respectively. Use duplicate plates for each dilution.

6. Incubate plates at 25°C for one week.

7. Re-weigh microcosms and incubate for two more weeks.

8. Examine plates from Period 1. The 2,4-D degrading colonies that arise on the indicator plates will appear black. Count the black colonies and calculate the mean number of 2,4-D degraders g^{-1} soil.

Third Period

Materials

2,4-D plates from Second Period

1. The 2,4-D degrading colonies that arise on the 2,4-D indicator plates will appear black. All colonies are presumptive 2,4-D degraders. Count plates with between 30 and 200 colonies.

2. Calculate the mean number of 2,4-D degrading colonies g^{-1} soil.

Fourth Period

1. Repeat the dilution and plating procedure from Period 2. Plate dilutions of 10^{-3}, 10^{-4}, and 10^{-5} to give final dilutions of 10^{-4}, 10^{-5}, and 10^{-6}.

Fifth Period

1. Count and calculate the mean number of 2,4-D degrading colonies g^{-1} soil.

10.4. TRICKS OF THE TRADE

DO:
- Mix 2,4-D thoroughly when amending the soil
- Monitor the screening broth to evaluate whether degradation is occurring

DO NOT:
- Forget to calculate the number of 2,4-D degrading bacteria on a dry weight of soil basis.

10.5. POTENTIAL HAZARDS

DO:
- Wear gloves when handling 2,4-D.
- Any media, or soil solutions containing 2,4-D will require special disposal. The TA will provide you with details.

10.6. QUESTIONS AND PROBLEMS

1. Construct a graph illustrating 2,4-D numbers in the control and amended soil over time.

2. If the screening broth changes color quickly what does this indicate?

3. If the screening broth changes color only after several weeks what does this indicate?

4. What are the implications of enriching in soil versus broth culture?

10.7. REFERENCES

Neilson, J.W., Josephson, K.L., Pepper, I.L., Arnold, R.G., DiGiovanni, G.D., and Sinclair, N.A. (1994) Frequency of horizontal gene transfer of a large catabolic plasmic (pJP4) in soil. *Applied and Environmental Microbiology* **60**, 4053–4058.

Newby, D.T., Gentry, T.J., and Pepper, I.L. (2000) Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. *Applied and Environmental Microbiology* **66**, 3399–3407.

EXPERIMENT 11

Adaptation of Soil Bacteria to Metals

11.1. OVERVIEW

Objective: ***To demonstrate the ability of a soil bacterial community to adapt to imposed metal stress.***

- Amend soil with Cd
- A non-amended soil is included as a control
- Incubate soil microcosms for two weeks
- Dilute and plate soils to determine the metal resistant populations and total heterotrophic bacteria after 0 and 2 weeks

11.2. THEORY AND SIGNIFICANCE

Metal contamination in the environment can arise from a number of activities, including mining and industrial wastes. Native soils and waters naturally contain metals, although these are generally in such low concentrations that they are not cause for concern. Metals, even biologically essential metals (Ca, Zn, Al) in high enough concentrations, can be naturally toxic to microorganisms. Metals can disrupt cell membranes, interfere with enzymatic reactions, denature proteins, and denature DNA. Consequently, microorganisms exposed to metals are forced to develop resistance mechanisms to protect cellular functions. Metals may also decrease microbial diversity and overall microbial numbers.

The discovery of microbial metal resistance has led to increased interest in the application of these mechanisms and the use of resistant organisms in the bioremediation of metal-contaminated systems. Metal resistance mechanisms include trapping of the metal in the bacterial exopolysaccharide layer; pumping the metal out of the system using ATP; and methylation, making the metal more volatile (Maier et al., 2000). It should be noted that metals cannot be degraded like organic compounds. Instead, metal toxicity can be reduced by making them less bioavailable or less soluble.

The effects of heavy metal exposure on soil microorganisms has been studied extensively for the last 40 years. Normally, soil microbial community response to heavy metal exposure has been measured by examining the effect on either diversity, activity, or culturable counts. Prior studies have usually indicated a decrease in overall diversity, activity, and many times culturable counts, in response to metal contamination (Kelly et al., 1999).

Many researchers have documented a similar increase in resistance to metals in soil bacteria that were isolated from contaminated soils. This

evidence suggests that during the shift in the bacterial community, sensitive species may be eliminated, while tolerant or metal resistant species may become dominant, and also that increased tolerance may arise as a result of exposure.

11.3. PROCEDURE

First Period

Materials
2 × 50 g soil
2 plastic cups for soil incubation
balance
weighing dishes
plastic wrap
rubber bands
marker pen
dissecting probe
0.6% stock solution of $Cd(NO_3)_2 \cdot 4H_2O$
9 peptone-yeast (PY) agar plates
9 peptone-yeast agar plates amended with Cd (500 µg Cd ml^{-1} agar)
1 sterile 95 ml water blank
4 sterile 9 ml water blanks
1 ml pipettes
glass hockey stick spreader
ethyl alcohol for flame sterilization
gas burner

1. Weigh out two fifty gram samples (dry weight basis) of soil.

2. To one sample of soil add Cd as Cd $(NO_3)_2$ to provide 500 µg of Cd g dry $soil^{-1}$ using a 6% stock solution of Cd $(NO_3)_2 \cdot 4H_2O$. This requires the addition of 1.14% ml of the stock solution to the soil.

3. Add 0.75 ml of 6% KNO_3 stock solution to the control soil to normalize soil nitrogen.

4. Adjust soil moisture content of the control and Cd amended samples to the value supplied by your instructor. Take into account the H_2O already added in Steps 2 and 3.

5. Using the one 95 ml and four 9 ml water blanks, dilute a 10 g sample of the control soil out to the 10^{-5} dilution.

6. Cover both samples with plastic wrap to reduce moisture losses, secure with a rubber band, and record the weights. Incubate at 25°C for two weeks.

7. Spread plate out the 10^{-3}, 10^{-4}, and 10^{-5} dilutions in triplicate on PY agar plates, using 0.1 ml of inoculum for each dilution. Note that the final dilutions will be 10^{-4}, 10^{-5}, and 10^{-6}. These plates will indicate total heterotrophic plate counts.

8. Similarly plate out the 10^{-1}, 10^{-2}, and 10^{-3} dilutions on PY agar plates amended with Cd (Final dilutions = 10^{-2}, 10^{-3}, and 10^{-4}). These plates will indicate numbers of Cd resistant bacteria initially present in the control soil.

9. Incubate all plates at 25°C for one week, prior to counting.

Second Period (t = 2 weeks)

Materials
incubated soils from first period
1 ml pipettes
glass hockey stick spreader
ethyl alcohol for flame sterilization
18 PY plates
18 PY + Cd plates
2 sterile 95 ml blanks
8 sterile 9 ml blanks

1. Re-weigh soil microcosms and adjust soil moisture content to original value. Be sure to include the weight of the plastic wrap and rubber band as in the first period.

2. Dilute 10 g samples of control soil in a similar manner to that of the first period. Similarly plate out on PY and PY + Cd amended agar plates.

3. Also dilute a 10 g sample of the Cd amended soil to the same dilutions i.e., through 10^{-5}. Spread plate out the 10^{-3}, 10^{-4}, and 10^{-5} dilutions onto PY plates in triplicate (Final dilutions = 10^{-4}, 10^{-5}, and 10^{-6}). Spread plate out the 10^{-1}, 10^{-2}, and 10^{-3} dilutions on PY + Cd plates (Final dilutions = 10^{-2}, 10^{-3}, and 10^{-4}).

4. Incubate all plates at 25°C for one week prior to counting.

11.4. TRICKS OF THE TRADE

DO:
- Add water to soil without stirring to avoid "puddling" the soil
- Plate from three successive dilutions to ensure getting countable numbers of colonies
- Change pipettes between plating control soil and Cd amended soil
- Ensure aerobic conditions during soil incubation

DO NOT:
- Allow soil to dry out during incubation—check weight after one week

11.5. POTENTIAL HAZARDS

DO:

- Wear gloves when handling Cd solutions or Cd amended soil
- Be careful to dispose of metal contaminated materials appropriately (ask instructor)

11.6. QUESTIONS AND PROBLEMS

1. Calculate concentration numbers of heterotrophic bacteria in control and Cd amended soils at time 0 and after 2 weeks incubation. Discuss changes in numbers.

2. Were there differences in colony morphology between isolates from i) control vs Cd amended soil; and ii) isolates obtained at time 0 vs 2 weeks?

3. Based on this experiment, did the soil microbial community "adapt" to the presence of Cd—discuss implications of your data?

11.7. REFERENCES

Kelly, J.J., Häggblom, M., and Tate, R.L. III. (1999) Changes in soil microbial communities over time resulting from one time applications of zinc: a laboratory microcosm study. *Soil Biology and Biochemistry* **31**, 1455–1465.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology.* Academic Press, San Diego.

Biodegradation of Phenol Compounds

EXPERIMENT 12

12.1. OVERVIEW

Objective: ***Determine the biodegradation rate of a synthetic phenol.***

- Add nutrients and bacteria to distilled water
- Add stock solutions of phenol and 2,4-dichlorophenol
- Incubate at 20°C
- Collect samples and determine oxygen concentration
- Graph rate of oxygen consumption against time of incubation

12.2. THEORY AND SIGNIFICANCE

Phenol is a naturally occurring compound in the environment and is readily biodegradable. Synthetically, phenol (Figure 12-1) is obtained from coal tar or by heating monochlorobenzene with aqueous NaOH under high pressure. It is used as a general disinfectant, in the manufacture of colorless or light-colored artificial resins, many medical and industrial organic compounds and dyes, and it is used as a reagent in chemical analyses.

Chlorinated phenols do not occur naturally in the environment, but have been manufactured and used extensively as herbicides/pesticides and preservatives. Examples of chlorinated phenols include 2,4-dichlorophenol, and pentachlorophenol (Figure 12-1). Both 2,4-dichlorophenol, and pentachlorophenol are prepared by chlorinating phenol; 2,4-dichlorophenol is a key intermediate in the manufacture of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D).

Pentachlorophenol is used as an insecticide for termite control, a pre-harvest defoliant, and general herbicide. It is also used as a preservative for wood, wood products, starches, dextrins, and glues.

In general, increased chlorination of organic compounds increases the recalcitrance or stability of the molecule in the environment. There are several reasons for recalcitrance of chlorinated phenols. One is that chlorine atoms simply block sites normally attacked by degrading enzymes, thus slowing biodegradation. A second reason is that chlorine atoms, which are electron-withdrawing groups, decrease the electron density on the aromatic ring. This slows enzymatic attack (hydroxylation) of the benzene ring, because attack occurs at sites of high electron density. A third reason for slower biodegradation is that chlorine constituents tend to decrease the water solubility of the phenol. Decreased water solubility results in decreased availability to microorganisms for biodegradation.

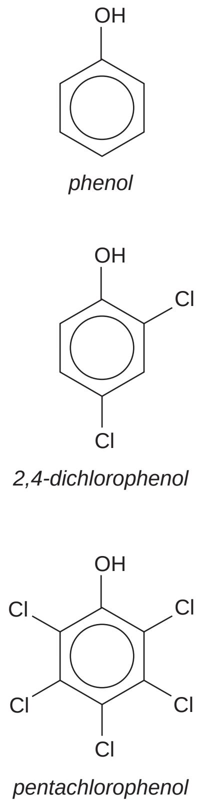


Figure 12-1 Phenol and some of its chlorinated derivatives.

12.3. PROCEDURE

Materials

BOD incubator at 20°C
1 1-L BOD dilution water[1]
3 10-ml pipettes
15 60-ml BOD bottles
1 Dissolved oxygen Test Kit[1] or oxygen probe
Bacterial inoculum (Polyseed)[2]
1 1-L beaker
2 500-ml beakers
3.5 ml stock phenol solution ($10\,g\,L^{-1}$)
3.5 ml stock 2,4-dichlorophenol solution ($10\,g\,L^{-1}$)[3]
compressed air supply

1. Prepare or obtain from the instructor one liter of BOD dilution water. BOD dilution water is prepared by the addition of Hach Chemical Company Buffer Nutrient Pillows to distilled water. The pillows contain phosphate buffer, $MgSO_4$, $FeCl_3$, $CaCl_2$, and glucose-glutamic acid.

2. Add 2 ml of polyseed to the BOD dilution water. The polyseed contains a mixture of bacteria capable of oxidizing biodegradable organic matter.

3. Split the one liter into three aliquots of approximately 333.3 ml.

4. Make blanks by filling 5 60-ml BOD dilution bottles with one aliquot.

5. To one of the aliquots, add 3.5 ml of stock phenol solution and mix well to give a phenol concentration of $100\,mg\,l^{-1}$ in the diluted mixture. Using the phenol-containing dilution water, fill five 60-ml BOD bottles (Figure 12-2). These are the phenol samples.

6. To the third aliquot, add 3.5 ml of stock 2,4-dichlorophenol solution and mix well to give a 2,4-dichlorophenol concentration of $100\,mg\,L^{-1}$ in the diluted mixture. Using this mixture, fill five 60-ml BOD bottles. These are the 2,4-dichlorophenol samples.

7. Determine the dissolved oxygen concentration in the blank, a phenol sample, and a 2,4-dichlorophenol sample.

8. Incubate the remaining BOD bottles in the dark at 20°C.

9. Withdraw sets of blank phenol and 2,4-dichlorophenol samples at 0 hour (prior to inoculation) and at 50, 170, 220, and 340 hours. Determine the amount of remaining dissolved oxygen in these samples.

---

1 Hach Company, Loveland, Colorado.

2 Hach Chemical Company. Dissolve 1 capsule in 500 ml dilution water. Aerate and stir for 60 min.

3 2,6-dichlorophenol may be substituted for 2,4-dichlorophenol.



Figure 12-2 BOD bottles used for this experiment. (Photo courtesy K.L. Josephson.)

12.4. POTENTIAL HAZARDS

DO:

- Wash your hand or skin immediately if they come in contact with phenol or 2,4-dichlorophenol

12.5. CALCULATIONS

Make a table of the dissolved oxygen concentration for each time point a sample was tested. Using graph paper plot the dissolved oxygen concentration for the blank, phenol samples and the 2,4-dichlorophenol samples with time. From these plots derive curves that show the course of dissolved oxygen uptake due to phenol and 2,4-dichlorophenol with time.

12.6. QUESTIONS AND PROBLEMS

1. Which compound degraded at a faster rate? Why was one degraded at a faster rate?

2. Why is it important to study the degradation of man-made compounds in the environment?

3. What conclusions can you make from the extent and rate of biodegradation of phenol and 2,4-dichlorophenol?

4. What is meant by recalcitrance? Give an example?

5. Why are chlorinated organic compounds more stable in the environment?

12.7. REFERENCES

Pitter, P., and Chudoba, J. (1990). *Biodegradability of Organic Substances in the Aquatic Environment,* pp. 165–177 and 251–266. CRC Press, Boca Raton.

APHA (1998) *Standard Methods for the Examination of Water and Wastewater.* American Health Association. Washington, DC.

Assimilable Organic Carbon

EXPERIMENT 13

13.1. OVERVIEW

Objective: ***To determine the amount of organic matter in drinking water available for bacterial growth.***

- Inoculate drinking water sample with *Pseudomonas fluorescens*
- Incubate for 7–9 days and assay on R_2A media
- Determine the amount of growth of *P. fluorescens*
- Calculate the amount of assimilable organic carbon in the water sample

13.2. THEORY AND SIGNIFICANCE

Growth of bacteria in finished drinking water can lead to the deterioration of water quality, violation of water quality standards (i.e., growth of coliform bacteria), and increased operating costs. Bacterial growth or regrowth in drinking water distribution and storage systems is defined as the multiplication of viable bacteria downstream from the treatment plant. Bacteria may grow on the inside of pipes forming a mat or biofilm. This results from viable bacteria passing through the disinfection process and being sustained by nutrients in the water. Other factors that influence regrowth include temperature, residence time in mains and storage units, and the efficacy of disinfection. Tests to determine the potential for bacterial regrowth focus on the concentration of nutrients.

Not all organic compounds are equally susceptible to microbial decomposition; the fraction that provides energy and carbon for bacterial growth has been called bacteriologically labile dissolved organic carbon, biodegradable organic matter, or assimilable organic carbon (AOC). It is estimated that the AOC in tap water is between 0.1 and 9% of the total organic carbon. However, this fraction may be higher if the treatment included ozonation, which enhances the bioavailability of refractory organic compounds to microorganisms. Easily measured chemical surrogates for AOC are not currently available. As alternatives to chemical methods, bioassays have been used.

The growth of a bacterial inoculum estimates the concentrations of limiting nutrients; the underlying assumptions are that nitrogen and phosphorus are present in excess, that organic carbon is limiting, and that the bioassay organism(s) represent the physiological capabilities of the system microflora. This procedure may use a defined inoculum of one to four species of bacteria.

Nutrient concentrations are estimated from changes in the maximum number of cells produced. Growth rate of the inoculum is determined from change in cell numbers, or incorporation of tritiated thymidine into bacterial

DNA. Cell densities or the flux of bacterial production are converted to AOC concentration by the growth yield of bacteria, defined as either the ratio between cells produced and organic carbon used, or biomass produced and organic carbon used.

The method described below is a single-species bioassay (van der Kooij et al., 1982), modified to reduce problems of bacterial and carbon contamination. It uses a defined inoculum and miniaturized incubation vessels, requires no specialized equipment, and has been related to the presence of coliforms in a drinking water distribution system. The single-species inoculum probably underestimates the total quantity of AOC. Other species, including a *Spirillum* designated strain NOX, which can utilize oxalate and other carboxylic acids, have been used in multispecies defined inocula. Critical aspects of the method, including the preparation of the incubation vessel, test water (Table 13-1), and inoculum and enumeration of the test organism, are transferable even if a different defined inoculum is used.

Table 13-1 Assimilable organic carbon levels of some common source waters

Source Water	AOC ($\mu g\,ml^{-1}$)
Biologically treated wastewater	3000–4300
River	62–128
Groundwater	<15

The AOC bioassay using *Pseudomonas fluorescens* strain P-17 involves growth to a maximum density of a small inoculum in a batch culture of pasteurized test water. Pasteurization inactivates native microflora. The test organism is enumerated by the spread plate method for heterotrophic plate counts and the density of viable cells is converted to AOC concentrations by an empirically derived yield factor for the growth of P-17 on acetate-carbon as a standard. The number of organisms at stationary phase is assumed to be the maximum number of organisms that can be supported by the nutrients in the sample, and the yield on acetate carbon is assumed to equal the yield on naturally occurring AOC.

In theory, concentrations of less than 1 μg carbon L^{-1} can be detected. In practice, organic carbon contamination during glassware preparation and sample handling imposes a limit of detection of approximately 5–10 μg AOC L^{-1}.

13.3. PROCEDURE

First Period

Materials

water sample
2 organic-carbon free borosilicate glass vials (45 ml capacity) with TFE-lined silicone septa[1]
0.7% (w/v) sodium thiosulfate
6 1-ml pipettes
pipette bulb
6 Petri dishes of R_2A media
10 9-ml blanks of sterile dilution water
1 stationary phase culture of *Pseudomonas fluorescens* P-17

1 Prepare bottles by washing with detergent in hot water, 0.1 *M* HCl, and deionized water, dry, cap with foil, and heat to 550°C for 6 h. Soak TFE-lined silicone septa in a 10% sodium persulfate (w/v) solution for 1 h at 60°C, rinse with carbon-free deionized water.

glass hockey stick spreader
ethyl alcohol for flame sterilization
gas burner
water bath at 70°C

Pseudomonas fluorescens can be grown in sodium acetate until it reaches the stationary phase of growth and stored up to 60 days at 5°C before use in AOC determinations. This prepared culture will be provided by the instructor.

1. Collect the water sample in two 45 ml vials. Fill each vial to the neck (40 ml) within as short a time as possible. Place septa on the vials within as short a time as possible and secure with screw caps. If the samples contain chlorine or other disinfectant, add 0.1 ml of a 0.7% solution of sodium thiosulfate.

2. Place the vials in a 70°C water bath for 30 min to inactivate any indigenous microbes.

3. Allow the vials to cool for 30 min, prior to inoculating with P-17 to give an initial inoculum concentration of 500 CFU ml^{-1}. To calculate the volume of inoculum needed to be added, the instructor should assay the innoculum R_2A media prior to the laboratory period, to determine the concentration of P-17 in the inoculum. Use Eq. 13-1 to calculate the volume of inoculum that should be added.

Second Period

Materials
incubated Petri dishes from Period 1
6 1-ml pipettes
pipette bulb
6 Petri dishes of R_2A media
buffered water
glass hockey stick spreader
ethyl alcohol for flame sterilization
gas burner

4. After 7 days determine the increase of P-17 by assay on R_2A media. Shake the vial vigorously for one min and remove 1 ml.

5. Dilute the sample in buffered water and plate three dilutions (10^{-2}, 10^{-3}, 10^{-4}) in duplicate.

6. Incubate the plates at room temperature for two days and count the bacterial colonies on each dilution.

Third Period

Materials
incubated Petri dishes from Period 2
6 1-ml pipettes
pipette bulb

6 Petri dishes of R_2A media
buffered water
glass hockey stick spreader
ethyl alcohol for flame sterilization
gas burner

7. After 9 days, repeat procedures 4–6.

Fourth Period

Materials
incubated Petri dishes from Period 3

8. Average the viable count results for the two assays and calculate the concentration of AOC using Eq. 13-2.

13.4. TRICKS OF THE TRADE

DO NOT:

- Contaminate the sample after inoculation of the *P. fluorescens*. Many types of bacteria can grow in water.

13.5. CALCULATIONS

Equation for the calculation of the volume of inoculum after assay on R_2A media:

$$\text{Volume of inoculum} = \frac{(500\,\text{CFU/ml}) \times (40\,\text{ml/vial})}{\text{CFU/ml stock inoculum}} \qquad (13\text{-}1)$$

The concentration of AOC is equal to the product of the mean of the viable counts and the inverse of the yield:

$$\frac{\mu\text{g AOC}}{\text{L}} = \frac{\text{mean CFU}}{\text{ml}} \times \frac{\mu\text{g acetate-C}}{\text{CFU}} \times \frac{1000\,\text{ml}}{\text{L}} \qquad (13\text{-}2)$$

When the empirical yield factor is used, the equation becomes:

$$\frac{\mu\text{g AOC}}{\text{L}} = \frac{\text{mean CFU}}{\text{ml}} \times \frac{\mu\text{g acetate-C}}{4.1 \times 10^{6}} \times \frac{1000\,\text{ml}}{\text{L}} \qquad (13\text{-}3)$$

or

$$\frac{\mu\text{g AOC}}{\text{L}} = \frac{\text{mean CFU}}{\text{ml}} \times \frac{2.44 \times 10^{-7}\,\mu\text{g acetate-C}}{\text{CFU}} \times \frac{1000\,\text{ml}}{\text{L}} \qquad (13\text{-}4)$$

When acetate is used as the carbon source in the determination of yield, AOC concentrations may be reported as acetate-carbon equivalents. Reporting AOC as μg carbon L^{-1} assumes that the yield on acetate is equal to the yield on naturally occurring AOC.

13.6. QUESTIONS AND PROBLEMS

1. What is coliform regrowth?

2. Why is control of biofilms important in the drinking water industry?

3. What is a biofilm?

4. Why do bacterial numbers increase after the ozonation of drinking water?

5. Why is it necessary to pasteurize the test water?

13.7. REFERENCES

LeChevallier, M.W., Babcock, T.M., and Lee, R.G. (1987) Examination and characterization of distribution system biofilms. *Applied and Environmental Microbiology* **53**, 2714–2724.

Van der Kooij, D., Visser, A., and Hijne, W.A.M. (1982) Determining the concentration of easily assimilable organic carbon in drinking water. *Journal American Water Works Association* **74**, 540–545.

Van der Kooij, D., Visser, A., and Oranje, J.P. (1982) Multiplication of fluorescent pseudomonads at low substrate concentrations in tap water. *Antonie van Leeuwenhoek* **48**, 229–243.

Biochemical Oxygen Demand

EXPERIMENT 14

14.1. OVERVIEW

Objective: ***To measure the amount of biodegradable organic matter in wastewater.***

- Prepare dilution water
- Prepare bacterial seed inoculum
- Prepare dilution water
- Make dilutions of wastewater to be tested
- Add bacterial seed
- Measure amount of oxygen in the dilutions
- Incubate the samples for 5 days at 20°C and measure amount of oxygen remaining
- Calculate the amount of BOD_5

14.2. THEORY AND SIGNIFICANCE

One of the main objectives of wastewater treatment is to reduce the organic content of wastewater. In order to assess the effectiveness of wastewater treatment processes, a method of measuring how much organic content has been removed is necessary. Measurement of biochemical oxygen demand (BOD) is one of the most important methods used by regulators and treatment plants to assess the effectiveness of wastewater treatment in reducing organic content (Bitton, 1999).

Biochemical (or biological) oxygen demand is the amount of dissolved oxygen in water consumed by microorganisms for the biochemical oxidation of organic and inorganic matter. The amount of oxygen being consumed can give a rough idea of how much organic matter is left in the water. If the amount of oxygen being used by organisms for the oxidation of organic matter decreases after wastewater treatment, it is assumed that the organic content has decreased also. Therefore, BOD is a useful measurement of the effectiveness of wastewater treatment. Conventional treatment removes up to 95% of BOD in wastewater. BOD values are also used to assess the impact that wastewater discharges will have on receiving waters. For example, discharged wastewater with high BOD could deplete the dissolved oxygen in receiving waters, harming fish and other organisms already in the receiving waters.

Total BOD is the sum of two types of BOD: carbonaceous and nitrogenous. Carbonaceous BOD is the amount of oxygen used by a mixed population of heterotrophic microorganisms to oxidize organic compounds. Nitrogenous BOD is the amount of oxygen used by autotrophic bacteria to oxidize NH_4^+ to nitrate. Nitrogenous BOD interferes with measurements of the oxygen

Table 14-1 Typical BOD values for different types of wastewater

Wastewater Type	BOD_5 (mg/L)
Raw domestic sewage	300
Domestic sewage after biological treatment	10
Slaughterhouse wastewater	2000
Distillery wastewater	30,000
Dairy wastewater	900
Rubber factory	3300
Tannery wastewater	1270
Raw textile dyeing wastewater	660
Textile wastewater after biological treatment	5
Raw draft mill effluent	226
Biologically stabilized kraft mill effluent	30

demand associated with organic content. Therefore, when measuring carbonaceous BOD, a chemical (2-chloro-6-(trichloromethyl) pyridine) is added to the water to inhibit nitrogenous BOD (Pipes and Zmuda, 1997; APHA, 1998).

In order to measure BOD, a water sample must contain a sufficient number of microorganisms to use the dissolved oxygen. Domestic wastewater, undisinfected wastewater effluents from treatment plants, and surface waters receiving wastewater discharges should contain these microorganisms. In contrast, disinfected waters may not contain a sufficient number of microorganisms. These waters need to be seeded with a culture of organisms. Commercially available pellets containing organisms commonly found in wastewater can be used (APHA, 1998).

BOD is measured as BOD_5, the oxygen demand over a 5-day incubation period. BOD in wastewater often exceeds the dissolved oxygen available in the water. Therefore, samples must be diluted in a solution of neutral phosphate. If water has been chlorinated, the chlorine must be neutralized so that the microbial population will survive. Dissolved oxygen in diluted samples (five dilutions are recommended) is measured with a membrane electrode or by the Winkler method, which involves titration. The samples are sealed in bottles and incubated at 20°C for 5 days. On day 5, the dissolved oxygen is measured again. BOD_5 can then be calculated using one of two equations, depending on whether the water requires seeding or not.

14.3. PROCEDURE

First Period

Materials
dark incubator at 20°C
2 L beaker of distilled water
20 10-ml pipettes



Figure 14-1 BOD bottles used in this experiment. (Photo courtesy K.L. Josephson.)

Hach Buffer Nutrient Pillows (containing phosphate buffer and nutrients)[1]
9 60-ml BOD bottles (Figure 14-1)
membrane electrode for measuring dissolved oxygen
PolySeed microorganism inoculum capsule[1]
2-chloro-6-(trichloromethyl) pyridine (TCMP)[1]
stir plate and stir bar
500 ml beaker
chlorine test kit, free & total, model CN-80[1]
DPD total chlorine reagent pillows[1]
0.1% sodium thiosulfate

Preparation of Samples

1. To make the dilution water, add the correct number of Hach Buffer Nutrient Pillows to 2 L of distilled water.

2. Take 10 ml of water sample and place in the Hach chlorine test kit tube.

3. Add a DPD reagent pillow to the tube. Follow the kit directions to determine the chlorine residual in the water.

4. Based on the chlorine residual, determine the amount of 0.1 N sodium thiosulfate[2] ($Na_2S_2O_3$) needed to neutralize the chlorine in the sample.

1 Hach Co., Loveland, Colorado.

2 25 g $Na_2S_2O_3 \cdot 5H_2O$ in 1 L distilled water.

Calculation:

$$\text{mL of } Na_2S_2O_3 \text{ needed} = \frac{RS}{(35{,}450)(N)}$$

where:

R = chlorine residual in mg/L
S = volume of water sample in ml
N = normality of the $Na_2S_2O_3$
35,450 = molecular weight in mg/L of chlorine

5. Add the required volume of $Na_2S_2O_3$ to the water sample.

6. If the water was chlorinated, it needs to be seeded.

For Water that Requires Seeding:

1. To make seed solution, break 1 PolySeed inoculum capsule and add contents to 500 ml of dilution water. Stir for 60 min (Figure 14-2).

2. Place 10, 15, 20, and 25 ml of seed solution in 4 BOD bottles. Bring the volume in each bottle up to 60 ml with dilution water (Figure 14-3).

3. To prepare dilution blanks, fill 5 BOD bottles with 52 ml each of dilution water. Make sure there is dissolved oxygen in the water by shaking each bottle vigorously (Figure 14-4).

4. To make dilutions, add 6 ml of sample to the first BOD bottle. Make serial dilutions by transferring 6 ml from each bottle to the next bottle (Figure 14-5).

5. Add 2 ml of the seed solution to each sample bottle (Figure 14-4).

6. Calibrate the membrane electrode according to the manufacturer's instructions.

7. Measure the dissolved oxygen in each seed and sample bottle with a membrane electrode within 30 minutes of making the dilution.

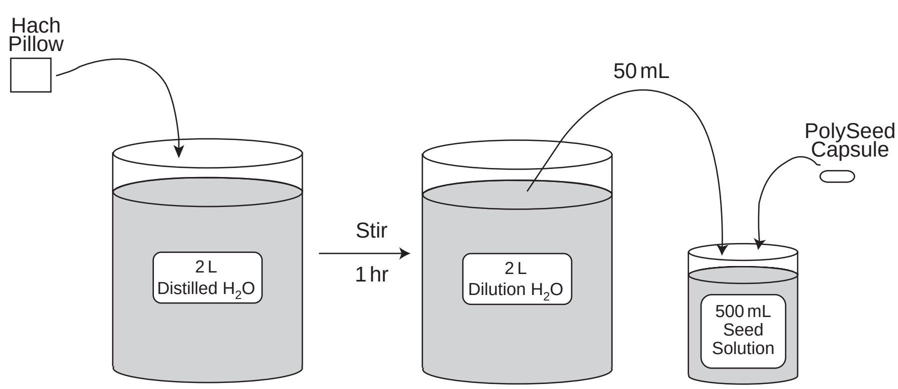


Figure 14-2 Preparation of dilution water and seed solution.

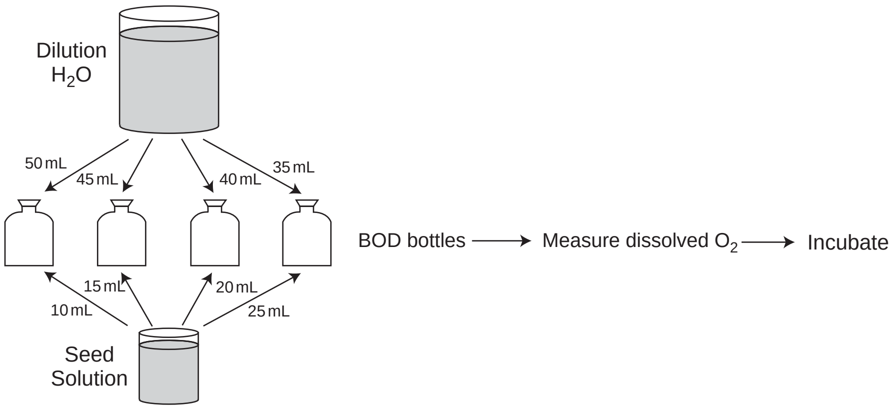


Figure 14-3 Preparation of seed controls.

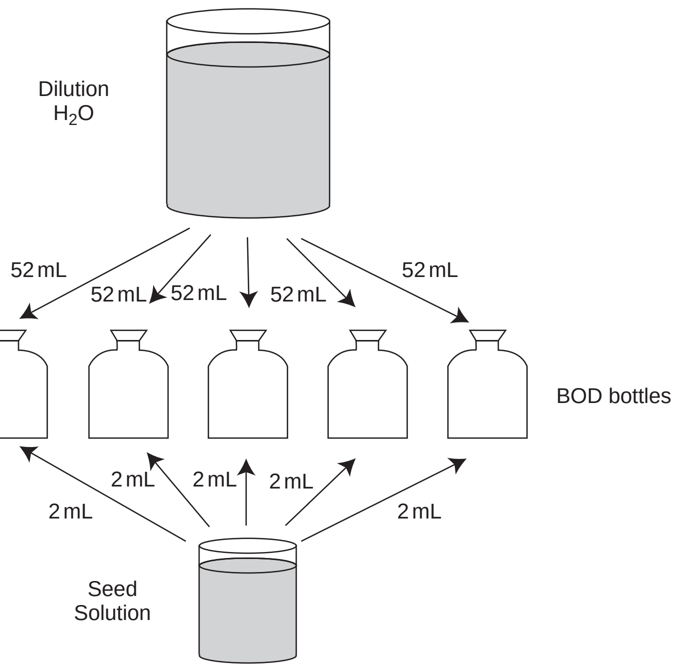


Figure 14-4 Preparation of seeded sample bottles.

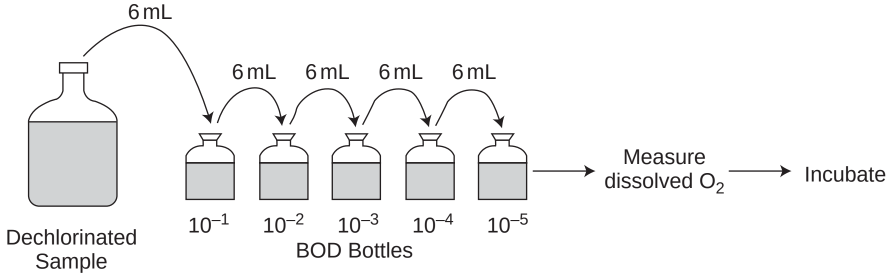


Figure 14-5 Sample dilutions.

8. If necessary, add additional dilution water to fill the bottles to the top, eliminating any air space. Seal the bottles tightly.

9. Wrap bottles in aluminum foil to block out light and incubate at 20°C for 5 days.

For Water that Does Not Require Seeding:

1. Place 300 ml of dilution water in a beaker. Add 3 mg of TCMP.

2. To prepare dilution blanks, fill 5 BOD bottles with 54 ml each of the dilution water prepared in step 1. Make sure there is dissolved oxygen in the water by shaking each bottle vigorously.

3. Measure the residual chlorine as described above and neutralize the water sample with the correct amount of sodium thiosulfate.

4. To make dilutions, add 6 ml of sample to the first bottle. Make serial dilutions by transferring 6 ml from each bottle to the next bottle.

5. Measure the dissolved oxygen in each bottle with a membrane electrode according to the manufacturer's instructions within 30 min of making the dilution.

6. If necessary, add additional dilution water to fill the bottles to the top, eliminating any air space. Seal the bottles tightly.

7. Wrap bottles in aluminum foil to block out light and incubate at 20°C for 5 days.

Second Period

Materials
membrane electrode
BOD bottles incubated for five days
BOD_5

1. Open each bottle and measure the dissolved oxygen with the membrane electrode.

14.4. TRICKS OF THE TRADE

<u>**DO**</u>:

- Be sure that you do not leave any air bubbles in the BOD bottles when you place the stopper in the bottle.

14.5. POTENTIAL HAZARDS

- Remember domestic wastewater (sewage) can contain pathogenic microorganisms, even after disinfection.

14.6. CALCULATIONS

To calculate BOD_5, use the bottles that have a final dissolved oxygen value of at least 1 mg/L and a dissolved oxygen depletion of at least 2.0 mg/L over 5 days ($D_1 - D_2 > 2.0$ mg/L).

For unseeded water:

$$BOD_5(\text{mg/L}) = (D_1 - D_2)/P$$

where:

D_1 = initial dissolved oxygen (mg/L) in the diluted sample
D_2 = dissolved oxygen (mg/L) in the diluted sample after 5 days of incubation
P = the decimal volumetric fraction of sample used (Example: 10^3 dilution = $1/10^3$, or 0.001)

Example:

The 1 : 100 dilution has the following values:

Initial dissolved oxygen = 4.2 mg/L
Dissolved oxygen after 5 days = 1.3 mg/L
Therefore, D_1 = 4.2 mg/L, D_2 = 1.3 mg/L, and P = 1/100, or 0.01
BOD_5 = (4.2 − 1.3)/0.01 = 290 mg/L

If the water is seeded, the BOD of the seed must be taken into account BOD_5 for seeded water is given by the equation:

$$BOD_5(\text{mg/L}) = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P}$$

where:

D_1 = initial dissolved oxygen (mg/L) in the diluted sample
D_2 = dissolved oxygen (mg/L) in the diluted sample after 5 days of incubation
P = the decimal volumetric fraction of sample used
B_1 = initial dissolved oxygen (mg/L) in the diluted sample
B_2 = dissolved oxygen (mg/L) in the diluted sample after 5 days of incubation
f = volume of seed solution in the water sample divided by the volume of seed in the seed control that has a final dissolved oxygen value of at least 1 mg/L and a dissolved oxygen depletion of at least 2.0 mg/L over 5 days ($B_1 - B_2 > 2.0$ mg/L).

Example:

The 1 : 10 dilution of sample has the following values:

Initial dissolved oxygen = 4.2 mg/L
Dissolved oxygen after 5 days = 1.1 mg/L
Therefore, D_1 = 4.2 mg/L, D_2 = 1.1 mg/L, and P = 1/10, or 0.1

The bottle containing 10 ml of seed solution has the following values:

Initial dissolved oxygen = 2.4 mg/L
Dissolved oxygen after 5 days = 1.0 mg/L
Therefore, B_1 = 2.4 mg/L, B_2 = 1.0 mg/L,

$$f = \frac{\text{volume of seed added to the water sample}}{\text{volume of seed in the bottle of seed solution used for calculation}} = \frac{2\,\text{ml}}{10\,\text{ml}} = 0.2\text{T}$$

$$\text{BOD}_5(\text{mg/L}) = \frac{(4.2-1.1)-(2.4-1.0)0.2}{0.1} = 28.2$$

14.7. QUESTIONS AND PROBLEMS

1. The major goal of sewage treatment is to reduce the amount of biodegradable carbon. Why is this necessary?

2. What is nitrogenous BOD? What is added to test sample to inhibit nitrogenous BOD?

3. Why do you need to neutralize chlorine before a BOD test?

4. Why is it necessary to dilute samples with expected high BOD levels?

5. Why is it important to leave no air bubbles in the BOD bottles during incubation?

14.8. REFERENCES

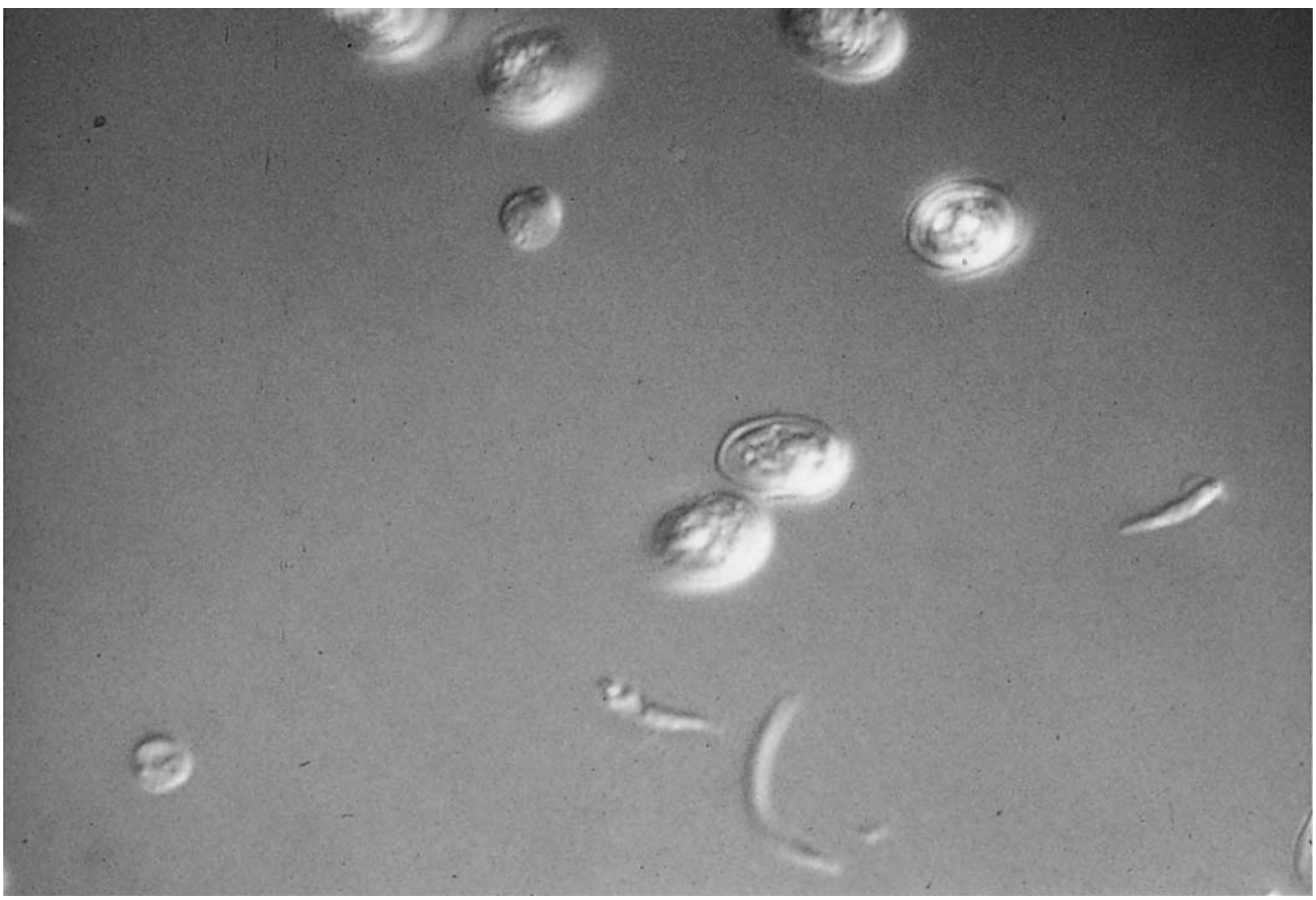
American Public Health Association (1998) Standard Methods for the Examination of Water and Wastewater, 20th edition. Washington, DC.

Bitton, G. (1999) *Wastewater Microbiology*, 2nd edition. Wiley-Liss, New York.

Pipes, W., and Zmuda, J. (1997) Assessing the Efficiency of Wastewater Treatment. In: *Manual of Environmental Microbiology,* 2nd edition. 2002. J. Hurst, G. Knudsen, M. McInerney, L. Stetzenbach, and M. Walter, eds., pp. 285–299. ASM Press, Washington, DC.

SECTION FOUR

Water Microbiology



Cryptosporidium with associated sporozoites.

Bacteriological Examination of Water: The Coliform MPN Test

EXPERIMENT 15

15.1. OVERVIEW

Objective: ***To detect coliform bacteria in water by the most probable number (MPN) method.***

- Inoculate water sample into LST lactose broth
- Observe tubes for gas production
- Confirm coliform detection by streaking of EMB and Endo agar
- Calculate coliform concentrations in the water via most probable number (MPN) procedure

15.2. THEORY AND SIGNIFICANCE

Microorganisms pathogenic to humans that are transmitted by water include bacteria (including blue-green algal toxins), viruses, and protozoa. Most of the microorganisms transmitted by water usually grow in the intestinal tract of man and leave the body in the feces. Fecal pollution of water used for swimming and drinking can then occur resulting in transmission of infectious microorganisms. The significance of this was recognized at the turn of the century when filtration and disinfection of drinking water was begun in the USA. This resulted in the almost complete elimination of waterborne cholera and typhoid in the country.

Routine examination of water for the presence of intestinal pathogens would be a tedious and difficult, if not impossible, task. It is much easier to demonstrate the presence of some of the nonpathogenic intestinal bacteria such as *Escherichia coli* and *Streptococcus faecalis*. These organisms are always found in the intestines and normally are not present in soil or water; hence, when they are detected in water, it can be assumed that the water has been contaminated with fecal material.

Coliform bacteria (of which *Escherichia coli* is a member) are often associated with enteric pathogenic organisms and have been shown to be useful indicators of the presence of fecal contamination.

Coliform bacteria occur normally in the intestines of humans and other warm-blooded animals and are discharged in great numbers in human and animal waste. In polluted water, coliform bacteria are found in densities roughly proportional to the degree of fecal pollution. When members of the coliform group are present, other kinds of microorganisms capable of causing disease also may be present.

Coliform bacteria are more hardy than disease-causing, non-spore-forming bacteria; therefore, their absence from water is an indication that the water is bacteriologically safe for human consumption. However, they are less sensitive than viruses and protozoan cysts to environmental factors (i.e., pH, temperature) and to disinfection. The presence of coliform bacteria, on the other hand, is an indication that disease-causing bacteria also may be present and that the water is unsafe to drink. In the United States drinking water regulations require the absence of coliform bacteria in 100 ml of potable water.

The coliform group includes all aerobic and facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria which ferment lactose with gas production in prescribed culture media within 48 hours at 35°C. Coliform bacteria include *Escherichia coli, Citrobacter, Enterobacter,* and *Klebsiella* species. An MPN test and the membrane filter test have been the methods most commonly used for the detection of coliforms in water. The membrane filter cannot be used easily with turbid waters because they will clog.

The MPN test for coliforms consists of three steps: a presumptive test, a confirmation test, and a completed test.

The first step is the presumptive test. A set of tubes of lauryl sulfate tryptose (LST) lactose broth is inoculated with samples of water and incubated (Figure 15-1). Lauryl sulfate is a surface-active detergent which inhibits the growth of gram-positive organisms while encouraging the growth of coliforms. Coliforms use any oxygen present in the broth and then ferment the lactose producing acid and gas under anaerobic conditions. Gas formation in 24 or 48 hours is a positive test. The formation of gas is observed by its presence in the inverted Durham tube (Figure 15-2).



Figure 15-1 A set of MPN tubes. (Photo courtesy K.L. Josephson.)



Figure 15-2 Inverted Durham tubes to collect gas. (Photo courtesy K.L. Josephson.)

Once it has been established that gas-producing lactose fermenters are absent in the water, it is presumed to be safe. However, gas formation may also be caused by noncoliform bacteria. Some of these, such as *Clostridium perfringens*, are gram-positive. To confirm the presence of gram-negative lactose fermenters, the next step is to inoculate media such as Levine's eosin methylene blue (EMB) agar, Endo agar, or brilliant green lactose bile (BGLB) broth from positive presumptive tubes in what is called the confirmed test.

Levine's EMB agar contains methylene blue, which inhibits gram-positive bacteria. Gram-negative lactose fermenters (coliforms) that grow on this medium will produce "nucleated colonies" (dark centers). *Escherichia coli* and *Enterobacter aerogenes* can be differentiated on the basis of size and the presence of a greenish metallic sheen. *Escherichia coli* colonies are small and

have this metallic sheen, whereas *E. aerogenes* colonies usually lack the sheen and are larger; differentiation in this manner is not completely reliable, however, *Escherichia coli* is the more reliable sewage indicator since it is not normally present in soil, while *E. aerogenes* has been isolated from grains and soil.

Endo agar contains a fuschsin sulfite indicator, which makes identification of lactose fermenters relatively easy. Coliform colonies and the surrounding medium appear red on Endo agar. Non-fermenters of lactose, on the other hand, are colorless and do not affect the color of the medium. In addition to these two media, there are several other media that can be used for the confirmed test. Brilliant green lactose bile broth (BGLB), Eijkman's medium, and EC medium are a few others that can be used.

BGLB broth, in addition to containing lactose, also contains two components inhibitory to gram-positive bacteria. Brilliant green is a dye related to crystal violet and belongs to the triphenylmethane dye series. Ox bile is a surface active agent which also inhibits the growth of gram-positive bacteria. Gas formation in 24 or 48 hours "confirms" the results of the presumptive step. The number of coliforms per 100 ml of water is then calculated from the distribution of positive and negative tubes in the test by referring to an appropriate table (such as Table 15-1). Results are reported as coliform MPN per 100 ml of water.

In some cases the organisms must be isolated and stained to provide the competed test. Positive BGLB tubes are streaked on eosin-methylene blue (EMB) agar. The two dyes, eosin and methylene blue, also inhibit the growth of gram-positive organisms. Typical colonies are isolated on nutrient agar slants and inoculated into LST broth. If gas is now formed by 24 or 48 hours, a Gram stain is made from the growth on the slant. If the cells are gram-negative after examination under oil and there is no indication of spores, the completed test is considered to be positive. Further biochemical studies (IMViC) may be performed on isolated cultures. In practice the competed test is seldom performed.

All three tests are necessary to prove than an organism in a water sample is in truth a coliform. In actual practice, when it has been shown that the presumptive and confirmed tests give essentially the same results, then the completed step is generally not done because of the time it takes.

15.3. PROCEDURE

First Period

Materials

water sample
3 test tubes containing Durham tubes and double strength LST lactose broth (DSLB)
6 test tubes containing Durham tubes and single strength LST lactose broth (SSLB)
1 10-ml pipette
1 1-ml pipette

Table 15-1 Most Probable Number (MPN) table used for evaluation of the data in this experiment, using three tubes in each dilution. The value printed **white on black** is referred to in the example in the Calculations section

Number of Positive Tubes in Dilutions				Number of Positive Tubes in Dilutions			
10 ml	**1 ml**	**0.1 ml**	**MPN per 100 ml**	**10 ml**	**1 ml**	**0.1 ml**	**MPN per 100 ml**
0	0	0	<3	2	0	0	9.1
0	1	0	3	2	0	1	14
0	0	2	6	2	0	2	20
0	0	3	9	2	0	3	26
0	1	0	3	2	1	0	15
0	1	1	6.1	2	1	1	20
0	1	2	9.2	2	1	2	27
0	1	3	12	2	1	3	34
0	2	0	6.2	2	2	0	21
0	2	1	9.3	2	2	1	28
0	2	2	12	2	2	2	35
0	2	3	16	2	2	3	42
0	3	0	9.4	2	3	0	29
0	3	1	13	2	3	1	36
0	3	2	16	2	3	2	44
0	3	3	19	2	3	3	53
1	0	0	3.6	3	0	0	23
1	0	1	7.2	3	0	1	39
1	0	2	11	3	0	2	64
1	0	3	15	3	0	3	95
1	1	0	7.3	3	1	0	**43**
1	1	1	11	3	1	1	75
1	1	2	15	3	1	2	120
1	1	3	19	3	1	3	160
1	2	0	11	3	2	0	93
1	2	1	15	3	2	1	150
1	2	2	20	3	2	2	210
1	2	3	24	3	2	3	290
1	3	0	16	3	3	0	240
1	3	1	20	3	3	1	460
1	3	2	24	3	3	2	1100
1	3	3	29	—	—	—	—

pipette bulb
incubator at 35°C

Presumptive Test

1. Set up three DSLB and six SSLB tubes as illustrated in Step 1 of Figure 16-3. Label each tube according to the amount of water that is to be dispensed to it: 10 ml, 1.0 ml, and 0.1 ml, respectively.

2. Mix the bottle of water to be tested by shaking 25 times.

3. With a 10 ml pipette, transfer 10 ml of water to the DSLB tubes.

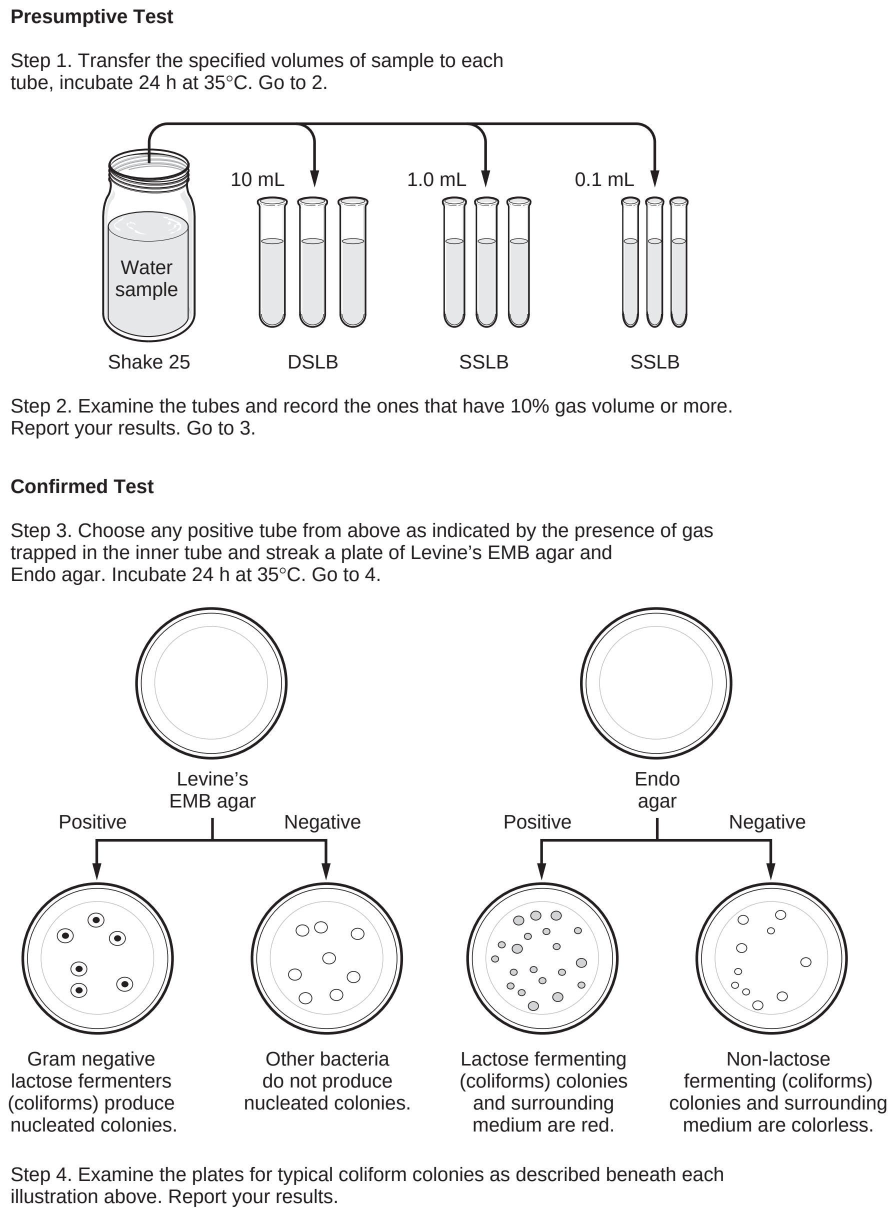


Figure 16-3 Procedure for performing an MPN test (presumptive and confirmed tests) for coliforms of water samples as described in this experiment.

4. With a 1.0 ml pipette, transfer 1 ml of water to each of the middle set of SSLB tubes and 0.1 ml to each of the last three SSLB tubes.
5. Incubate the tubes at 35°C for 48 h.

Second Period

Materials

incubated tubes from the previous week
1 Petri plate of Levine's EMB agar
1 Petri plate of Endo agar

inoculation loop
gas burner

Presumptive Test

1. Examine the tubes and record the number of tubes in each set that have a gas bubble(s) in the inverted Durham tube. Determine the MPN by referring to Table 15-1. See the Calculations section for an example of the calculations.

2. Record this data for your report.

Confirmed Test

1. Select one positive lactose broth tube from the presumptive test and streak one plate of each of Levine's EMB agar and Endo agar (Step 3 in Figure 15-1). Use a streak method which will produce good isolation of colonies such as that described in Figure 5-2 of Experiment 5, "Bacteria and Actinomycetes." If all your tubes were negative, borrow a positive tube from another student.

2. Incubate the plate for 24 h at 35°C.

Third Period

Materials
incubated plates from the previous week

1. Look for typical coliform colonies on both kinds of media. Record your results for your report. If no coliform colonies are present, the water is considered safe to drink.

15.4. TRICKS OF THE TRADE

DO:
- Label your dilutions

15.5. CALCULATIONS

Consider the following: If you had gas in the first three tubes and gas only in one tube of the second series, but none in the last three tubes, your test would be read as 3-1-0. Table 10-1 indicates that the MPN for this reading would be 43. This means that this particular sample of water would have approximately 43 organisms per 100 ml with 95% probability of there being between 7 and 210 organisms. Keep in mind that the MPN of 43 is a statistical probability number.

15.6. QUESTIONS AND PROBLEMS

1. Determine the concentration of coliforms per 100 ml in the water sample tested.
2. Why use coliforms instead of directly testing for pathogens?
3. Name the two most prominent species of coliforms.
4. What is the definition of coliforms?
5. Name four diseases that are waterborne.
6. What genus of bacteria are included in the coliform group?
7. What color do coliform bacteria appear on mEndo agar?
8. Why is ox bile added to BGLB broth?
9. What is the purpose of the Durham tube?
10. What is the coliform standard in the USA for drinking water?

15.7. REFERENCE

APHA (1998) *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.

Membrane Filter Technique

EXPERIMENT 16

16.1. OVERVIEW

Objective: ***To detect coliform and fecal coliform bacteria in water by the membrane filtration method.***

- Filter water samples through membrane filters
- Incubate membrane filters on media agar for 24–48 hrs
- Observe and count colonies of coliform and fecal coliform bacteria
- Calculate coliform and fecal coliform bacteria in water

16.2. THEORY AND SIGNIFICANCE

In addition to the MPN technique, a method utilizing a membrane filter is commonly used for detecting and quantifying bacteria in water.

A measured amount of water is filtered through a membrane with a pore size of about 0.45 µm, which traps the bacteria on its surface. The membrane is then placed on selective agar or a thin absorbent pad that has been saturated with a medium designed to grow or permit differentiation of the organisms sought—a modified Endo medium, for example, if coliform bacteria are sought. Fecal streptococci may be selected by use of a modified Enterococcus-agar medium (KF agar) containing azide, and fecal coliform medium (mFC broth) with incubation at 44.5°C. After incubation in a small Petri dish, the colonies are counted under low magnification.

The success of this method depends on using effective differential or selective media that will enable easy identification of colonies. This method has advantages over the traditional MPN water analysis because it is more direct and quicker (giving results in 18–24 hours) and can easily test large volumes of water (hence yielding more accurate results). The method cannot be used with highly turbid waters as the filter may clog.

As applied to the membrane filter technique, the coliform group may be defined as comprising all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that develop a red colony with a metallic green sheen within 24 hours at 35°C on an Endo-type medium containing lactose. When purified cultures of coliform bacteria are tested they produce a negative cytochrome oxidase (CO) and positive β-galactosidase (ONPG) reaction. Generally, all red, pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms by this technique.

For many years, the total coliform group served as the main indicator of water pollution. However, because many of the organisms in this group are not limited to fecal sources, methods were developed to restrict the

enumeration to those coliforms which are more clearly of fecal origin. A modified method devised by Eijkman called for a higher incubation temperature, and this was refined further to distinguish what are known as thermotolerant coliforms (also referred to as fecal coliforms). This indicator group has all the properties of the total coliform group and, in addition, is able to ferment lactose with the production of gas in 24 hours at 44.5°C. In general, this test enumerates organisms of the genera *Escherichia* and *Klebsiella*. *Klebsiella* of non-fecal origin may occur in nutrient-rich, non-fecal sources, such as pulp mill effluents. In the United States fecal coliforms have been used for acceptability of bathing waters.

The membrane filter (MF) procedure for fecal coliforms uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity and gives 93% accuracy in differentiating between coliforms found in the feces of warm-blooded animals and those from other environmental sources. Because incubation temperature is critical, MF cultures are submerged in a waterproofed enclosure (plastic bag, i.e., Ziploc®) in a water bath for incubation at the elevated temperature. Alternatively, an appropriate, accurate solid heat sink incubator may be used.

16.3. PROCEDURE

First Period

Materials
forceps
ethyl alcohol for flame sterilization
gas burner
6 sterile 50 × 12 mm Petri dishes with tight cover
6 sterile 0.45 μ pore, 47 mm diameter membrane filters with pads
1 nonsterile 1 liter filter flask
1 filter unit, graduated, with top, sterile
6 ml mEndo broth-MF, sterile
15 ml mFC agar, sterile
1 sterile 10 ml pipette
1 pipette bulb
1 flask with 200 ml distilled water, sterile
thermostatically controlled water bath at 44.5°C
resealable plastic bag for water bath large enough to hold the plates
vacuum hoses for the filter
vacuum source

1. Flame a pair of forceps and place a sterile blotter pad from the sterile filter-pack in the bottoms of 3 Petri plates.

2. Pipette 2 ml of mEndo broth-MF onto each pad and replace covers.

3. Pipette 5 ml of melted mFC agar into the bottoms of each of 3 Petri plates and let solidify.

4. Assemble the filter funnel on the flask (Figure 16-1).



Figure 16-1 Filtration apparatus needed in this experiment. (Photo courtesy K.L. Josephson.)

5. Remove the funnel top and with an alcohol-flamed forceps, place a sterile membrane filter in place with the grid side up and center it. Place the funnel top being careful not to tear the filter. Use a clamp to hold the assembly together.

6. Carefully pour about 50 ml of sterile distilled water into the funnel. Then pipette 1 ml of the water sample into the funnel. Return the pipette to the water sample and let it stand there.

7. Apply the vacuum gently. Just as the liquid level approaches the filter, rinse the sides with a small amount of the sterile distilled water, and let the vacuum draw all of the water through the filter.

8. Unclamp the funnel top with the vacuum still applied. With a sterile forceps, remove the filter and carefully "roll" onto the pad of mEndo broth-MF. Avoid trapping bubbles of air. Label the plate "1 ml."

9. Repeat step numbers 5, 6, 7, and 8, finally transferring the filter to one of the mFC plates labeled "1 ml."

10. Repeat step numbers 5, 6, 7, and 8, this time using 10 ml of water sample instead of 1 ml. Label this plate "10 mL."

11. Repeat step numbers 5, 6, 7, and 8 one more time for the mFC plate. Label this "10 ml."

12. Now place a filter on the funnel block and clamp the funnel down gently. Pour the water sample into the funnel (no vacuum) until the meniscus is at the 100-ml mark.

13. Apply the vacuum and rinse the sides with sterile distilled water until the water is drawn through.

14. Remove the funnel and transfer the filter to the last mEndo broth-MF plate. Label it "100 ml."

15. Repeat step numbers 12, 13, and 14, transferring the filter to the last mFC plate labeled "100 ml."

16. Incubate the mEndo broth-MF plates at 35°C with the cover up for 24 h.

17. Insert the mFC plates inverted (bottom up) into a water-tight, resealable plastic bag and incubate at 44.5°C for 24 h immersed in a thermastatically controlled water bath.

Second Period

Materials
incubated plates from Period 1
dissection microscope with 10–15× magnification

1. Examine the mEndo broth-MF plates using a low power (10–15× magnification) dissection microscope. Coliform colonies are red or pink showing a bright green metallic sheen. Colonies without the green sheen are non-coliforms. Count the coliform colonies and record the results in the form provided.

2. Examine the mFC plates in the same fashion. Fecal coliform colonies are blue regardless of shade. All others are not coliforms. Count and record your results.

3. Note that coliform results are usually reported "per 100 ml" rather than "per milliliter."

16.4. TRICKS OF THE TRADE

DO:

- Be careful when handling the filters with the forceps. The filters are very fragile and are easily torn

- Allow the forceps to cool after flaming
- Avoid air bubbles by rolling the filter onto the media pad

DO NOT:
- Do not bring the filter near an open flame as it will ignite and quickly burn

16.5. POTENTIAL HAZARDS

DO:
- Keep the forceps away from the ethyl alcohol. Drops of ignited alcohol may fall into the beaker of ethyl alcohol igniting it.

16.6. CALCULATIONS

The bacterial count per 100 ml is calculated as follows:

$$\frac{\text{count}}{100\,\text{ml}} = \frac{\text{count on filter}}{\text{ml filtered}} \times 100$$

16.7. QUESTIONS AND PROBLEMS

1. What are the limitations of the membrane filter method?

2. Do coliforms, even fecal coliforms, always mean fecal pollution?

3. What is the difference between a coliform and fecal coliform?

4. Fecal coliform bacteria have been used to set standards for what types of waters?

5. What are the advantages of the membrane filter method over the MPN method?

16.8. REFERENCE

APHA (1998) *Standard Methods for the Examination of Water and Wastewater*, 2nd edition. American Public Health Association, Washington, DC.

Defined Substrate Technology for Detection of Coliforms and Fecal Coliforms

EXPERIMENT 17

17.1. OVERVIEW

Objective: ***Detection of total coliforms and Escherichia coli at the same time in water.***

- Inoculate water samples with a defined substrate that will detect both total coliforms and *E. coli*
- Incubate 24–48 hours at 35 ± 0.5°C
- Determine presence of total coliforms by media color change and *E. coli* by fluorescence in the presence of ultraviolet light

17.2. THEORY AND SIGNIFICANCE

More rapid and simple methods for the detection of indicator bacteria in water have long been sought. **Defined substrate technology** (**DST**) is a new approach for the simultaneous detection, specific identification, and confirmation of total coliforms and *Escherichia coli* in water. Colilert® was the first commercial DST test to receive U.S. Environmental Protection Agency approval for drinking water analysis. This test uses specific indicator nutrients ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG) (Figure 17-1). A water sample is incubated with the Colilert® reagent for 24 hours. If a coliform is present, indicator nutrient is hydrolyzed by the enzyme β-galactosidase of the organism, thereby releasing the indicator portion, ortho-nitrophenyl (ONPG). The free indicator imparts a yellow color to the solution. *E. coli* possess an additional constitutive enzyme, glucuronidase, that hydrolyzes the second indicator nutrient, MUG. As a result of this hydrolysis, MUG is cleaved into a nutrient portion (glucuronide), which is metabolized, and an indicator portion, methylumbelliferone, which fluoresces under ultraviolet light. Thus, two separate and specific microbial assays are carried out simultaneously with the same sample. At low levels (1 CFU $100\,ml^{-1}$), total coliforms and *E. coli* can be detected simultaneously in potable and other waters in 24 hours. These systems can also be used in a MPN test.

Regulations for drinking water in the United States also allow for a presence-absence (P–A) test (Figure 17-2). These regulations require that coliforms be absent in 100 ml of drinking water. In the P–A test, 100 ml of drinking water is added to a bottle containing the substrate in either powder or liquid form. The bottle is then incubated at 35 ± 0.5°C for 24 hours.

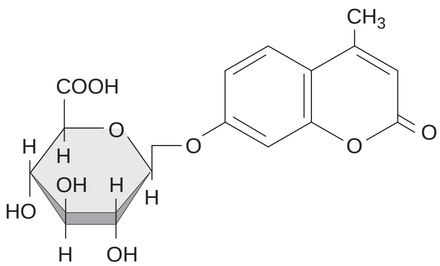


Figure 17-1 The structure of 4-methylumbelliferyl-β-D-glucuronide (MUG).

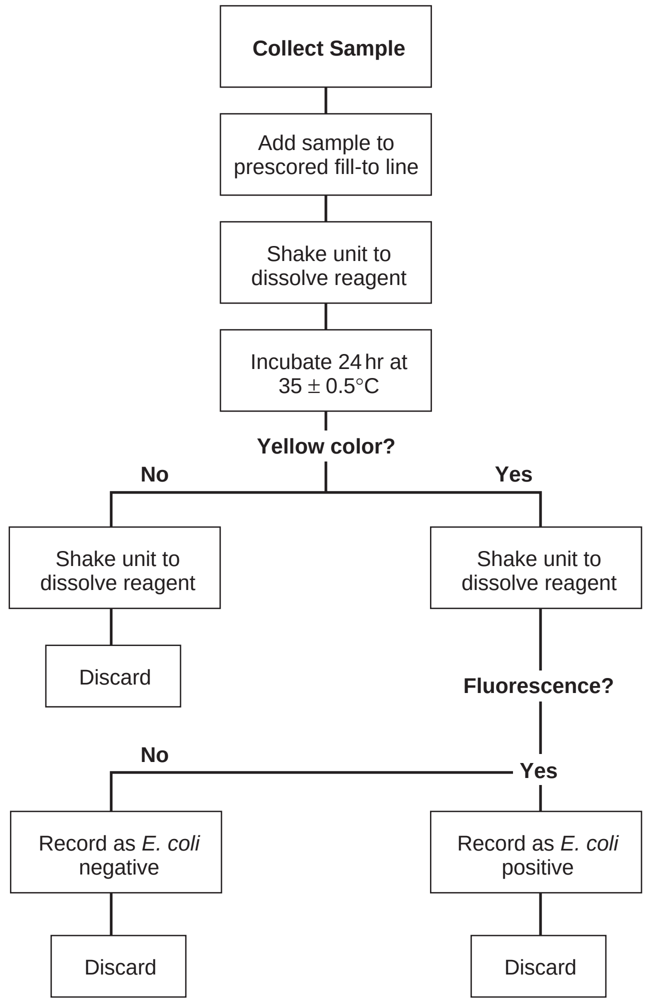


Figure 17-2 Flow chart for determining the presence or absence (P–A) of total coliforms and *E. coli* in water samples.

17.3. PROCEDURE

First Period

Materials

30 ml water sample
9 tubes of Colilert® powdered media
290 ml sterile dilution water blanks
3 10-ml pipettes
pipette bulb
incubator at 35 ± 0.5°C
1 100-ml sample bottle of transparent, non-fluorescent borosilicate glass containing 1 ml 1% (w/v) sodium thiosulfate

Colilert® MPN

1. Label 3 tubes of Colilert® powdered media undiluted and add 10 ml of water sample to each tube.

2. Cap the tubes tightly and mix vigorously to dissolve the powdered reagent by repeated inversion. Some reagent particles may remain undissolved, but this does not effect the test.

3. Label another set of 3 tubes "1 : 10."

4. Dilute the original sample of water by placing 10 ml into a 90 ml dilution blank bottle. Cap and shake vigorously to mix.

5. Inoculate 10 ml each of the 1 : 10 dilution into the 3 tubes labeled 1 : 10 and mix by shaking.

6. Label another set of 3 tubes "1 : 100."

7. Add 10 ml of the 1 : 10 dilution bottle to another 90 ml dilution blank bottle to yield a 1 : 100 dilution.

8. Inoculate 10 ml each of the 1 : 100 dilution into 3 tubes labeled 1 : 100 and mix by shaking.

9. Incubate tubes at 35 ± 0.5°C for 24 h.

Colilert® P–A Test

1. A 100 ml sample bottle will be provided. Students will be required to collect a water sample the evening or morning before the next laboratory section in this bottle. The sterile bottle contains 1 ml of 1% sodium thiosulfate solution to neutralize the presence of any chlorine which may be present in the sample.

Second Period

Materials
incubated Colilert® tubes from Period 1
100 ml water sample collected since Period 1
1 tube of Colilert® reagent
1 long-wavelength (365 nm) ultraviolet lamp
UV-protective goggles
secluded area for safe UV viewing
incubator at 35 ± 0.5°C

Colilert® MPN

1. Read the tubes within 24–28 h. The presence of an intense yellow color indicates the presence of total coliforms. The samples are considered negative for total coliforms if no color is observed after 24 h. If the

yellow color is present, observe the sample for fluorescence by placing the tubes two inches from a long-wave ultraviolet lamp. An intense fluorescence indicates the presence of *E. coli*.



Figure 17-3 A tube of Colilert® reagent is being added to a water sample in a sterile, non-fluorescent container. The container to the right has turned yellow after incubation at 35 ± 5°C for 24–28 h, thus testing positive for total coliforms.

Colilert® P–A Test

1. Aseptically open a tube of Colilert® reagent and add the contents to a 100 ml water sample in a sterile, transparent, non-fluorescent borosilicate glass container or equivalent (Figure 17-3). Aseptically cap and seal the vessel.

2. Shake vigorously by repeated inversion to aid dissolution of the reagent. Some particles may remain undissolved. Dissolution will continue during incubation.

3. Incubate reagent/sample mixture at 35 ± 0.5°C.

Third Period

Materials
incubated Colilert® tubes from Period 2
1 long-wavelength (365 nm) ultraviolet lamp

Colilert® P–A Test

1. Read the tubes with 24–28 h. The presence of a yellow color indicated the presence of total coliforms. If the yellow color is present observe the sample for fluorescence by placing the tubes two inches from a long-wave ultraviolet lamp. An intense fluorescence indicates the presence of *E. coli*.

17.4. TRICKS OF THE TRADE

<u>DO</u>:
- Label all tubes carefully

17.5. POTENTIAL HAZARDS

<u>DO NOT</u>:
- Do not observe tubes or bottles under UV light without protective goggles. The wavelengths of UV light involved are harmful to the eyes.

17.6. CALCULATIONS

By examining the MPN table, Table 17-1 of Experiment 17, "Bacteriological Examination of Water: The Coliform MPN Test," calculate the total coliform and *E. coli* concentrations in the water samples.

17.7. QUESTIONS AND PROBLEMS

1. What does MUG mean?

2. What are the advantages and disadvantages of a P–A test?

3. Why is it important to determine both total coliforms and *E. coli* in water?

17.8. REFERENCES

Covert, T.C., Shadix, L.C., Rice, E.W., Haines, J.R., and Feyberg, R.W. (1989) Evaluation of the autoanalysis Colilert® test for detection and enumeration of total coliforms. *Applied and Environmental Microbiology* **55**, 2433–2447.

Edberg, S.C., Allen, M.J., Smith, D.B., and The National Collaborative Study. (1988) National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: Comparison with the standard multiple tube fermentation method. *Applied and Environmental Microbiology* **54**, 1595–1601.

Film Medium for the Detection of Coliforms in Water, Food, and on Surfaces

EXPERIMENT 18

18.1. OVERVIEW

Objective: ***To determine the concentration of bacteria on surfaces using media bound to a plastic film.***

- Hydrate film bound media
- Sample selected surfaces for *E. coli* and coliform bacteria
- Incubate film for 24 h
- Identify and count colonies of *E. coli* and coliform bacteria

18.2. THEORY AND SIGNIFICANCE

Detection of bacteria on surfaces is important in the evaluation of sanitation programs in the food and medical industries. Food-contact surfaces and equipment play a major role in the microbiological quality of finished food products. Surfaces which come in contact with a food product must be periodically sampled to determine the level of microorganisms present. Failure to do so may jeopardize the taste and shelf life of the product, as well as the health of the consumer. Proper cleaning and disinfection of surfaces is also important to prevent the transmission of pathogens in day care centers and hospitals.

Two standard environmental monitoring procedures for the detection of coliforms and other bacteria on surfaces are the direct contact plate (also called Rodac plates) and the swab method. Rodac plates are small Petri dishes which contain agar poured until a convex surface forms. The hardened agar surface is then pressed firmly against the surface to be sampled. In the swab technique, a sterile cotton swab premoistened with buffered saline or letheen broth is passed over the surface to be sampled. The swab head is then placed in a tube of sterile solution of buffered saline or broth and broken off. The tube is vortexed and the solution in the tube assayed by spread plate or pour plate methods.

Petrifilm™ is a dry media (hydrated) bound to a polyethylene coated paper printed with a grid which can be used for monitoring the microbial quality of surfaces. Petrifilm™ plates can also be used for sampling surfaces or 1 ml volumes of milk, water, or other fluids. Petrifilm™ plates eliminate the need for media preparation and autoclaving and can be stored for prolonged periods before use.

18.3. PROCEDURE

First Period

Materials
2 Petrifilm™ coliform count plates
2 1-ml pipettes
pipette bulb
2 ml of sterile nutrient broth or 0.1% (w/v) peptone water
incubator set at 35 ± 0.5°C
food or water sample prepared in the appropriate manner

Hydrating a Petrifilm™ Plate

1. Place a Petrifilm™ plate on a flat surface.

2. Lift the top film and dispense 1 ml of sterile nutrient broth or 0.1% peptone onto the center of the bottom film (Step 1, Figure 18-1).

3. Replace the top film down onto the diluent using a rolling motion so as not to entrap air bubbles (Steps 2 and 3, Figure 18-1).

4. Distribute the diluent with a downward pressure on the center of the plastic spreader (recessed side down). Do not slide the spreader across the film (Step 4, Figure 18-1).

5. Remove the spreader using a vertical motion (Step 5, Figure 18-1) and leave the plates undisturbed for 1 min to permit solidification of the gel.

6. Allow a minimum of 30 min for the gel to completely solidify before using the plate for surface sampling.

Surface Sampling Procedure

7. Lift the top film of the prehydrated Petrifilm™ plate (gel will adhere to the top film).

8. Place the gel and top film in contact with the surface to be sampled.

9. Firmly rub your fingers over the entire film side of the gelled area to ensure good contact with the surface.

10 Lift the film from the surface and rejoin the top and bottom sheets of the Petrifilm™.

11. Incubate the plates in a horizontal position with the clear side up at 35 ± 0.5°C for 24 h.

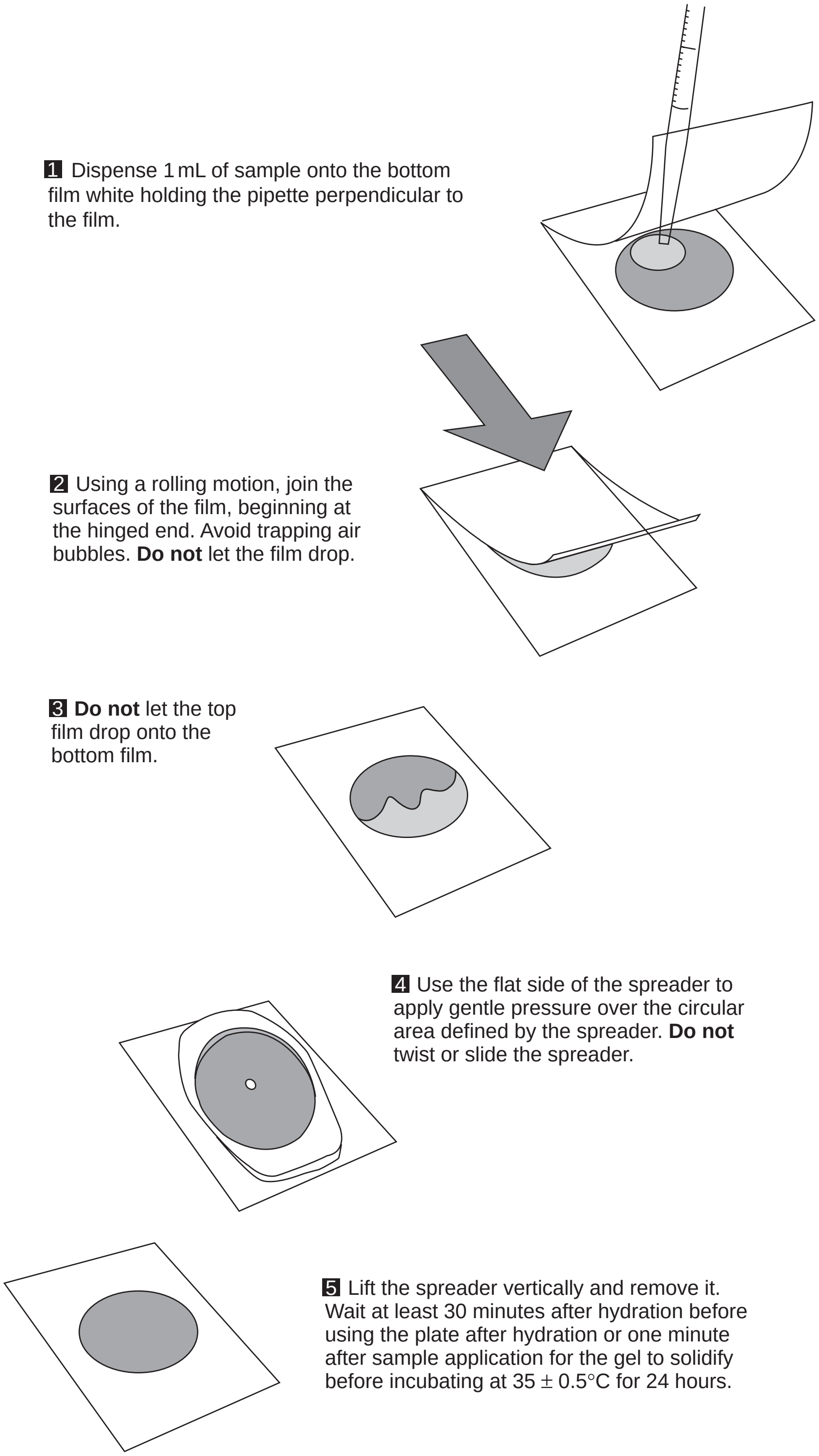


Figure 18-1 Procedure for hydrating and applying a water sample to a Petrifilm™ plate.

Water or Food Sampling

12. Place Petrifilm™ on a flat surface. Lift the top film.

13. With pipette perpendicular to Petrifilm™, dispense 1 ml of sample onto center of bottom film (Step 1, Figure 18-1).

14. Using a rolling motion, lower the top film so as not to entrap air bubbles. Do not let the top film drop (Steps 2 and 3, Figure 18-1).

15. With the flat side down, place the spreader on the top film over inoculum (Step 4, Figure 18-1). Gently apply pressure on the spreader to distribute inoculum over the circular area defined by the spreader. Do not twist or slide the spreader.

16. Lift spreader using a vertical motion. Wait 1 min for the gel to solidify (Step 5, Figure 18-1).

17. Incubate at 35 ± 0.5°C for 24 h.

Second Period

Materials
incubated plates from Surface Sampling, Period 1
incubated plates from Water or Food Sampling, Period 1

Surface Sampling

1. Remove the plates from the incubator and record the number of coliforms and *Escherichia coli*. The indicator contained in the Petrifilm™ reacts with β-glucuronidase produced by *E. coli* to form a blue precipitate in the medium. All blue colonies (regardless of gas production) are counted as *E. coli*. Coliforms ferment the lactose in the medium to produce gas which is trapped between the films of the plate. All colonies associated with gas bubbles (bubbles are less than one colony diameter from the colony) are counted as coliforms. Red colonies without gas bubbles are other Gram-negative organisms.

   Coliform and *E. coli* bacteria can be found routinely on surfaces in public restrooms, such as the bottom of toilet seats, sinks, taps, and floor.

Water or Food Sampling

1. Remove the plates from the incubator and record the results as above. The number of *E. coli* or coliform bacteria is the amount present in 1 ml of the original sample.

18.4. TRICKS OF THE TRADE

DO:

- Practice good aseptic technique throughout the prehydration procedure to avoid introducing any contamination

DO NOT:

- Touch the bacterial growth area with your fingers

18.5. QUESTIONS AND PROBLEMS

1. Why is it important to test surfaces for the presence of bacteria?
2. What is a possible source of *E. coli* on surfaces?
3. What produces the blue *E. coli* colonies on the Petrifilm™?

18.6. REFERENCE

Gerba, C.P., Wallis, C., and Melnick, J.L. 1975. Microbial hazards of household toilets. Droplet production and the fate of residual organisms. *Applied and Environmental Microbiology* **30**, 229–237.

Detection of Bacteriophages

EXPERIMENT 19

19.1. OVERVIEW

Objective: ***To detect coliphages in water/sewage.***

- Incubate water or sewage sample with host bacteria
- Incubate 24–48 hrs
- Count viral plaques in the bacterial lawn
- Calculate the number of coliphage in the sample

19.2. THEORY AND SIGNIFICANCE

Viruses are ultramicroscopic—too small to be viewed with the light microscope, visible only with the greater resolution of the electron microscope. They are particulate, not cellular, being more or less macromolecules composed primarily of a nucleic acid genome, either DNA or RNA, and protein. They are obligate intracellular parasites whose nucleic acid genomes control and utilize the synthetic capacities of their host cells for replication.

Viruses which infect the intestinal tract of humans and animals are known as enteric viruses. They are excreted in feces and can be isolated from domestic wastewater. Viruses which infect bacteria are known as bacteriophage, and those which infect coliform bacteria are called coliphage. The phages of coliform bacteria are found anywhere coliform bacteria are found.

Basically, the viral particle, or virion, is a nucleic acid core surrounded by a protein coat, or capsid composed of protein subunits or capsomers. In some more complex viruses, the nucleocapsid is surrounded by an additional envelope and some have spike-like surface appendages or tails. Concentrations of human viruses in raw sewage range from 10^3–$10^7\,L^{-1}$. Concentration of coliphages in raw sewage ranges from 10 to 100 per ml.

Some coliphage may have complex structures consisting of a head (capsid), which contains the nucleic acid core, a tail, and tail fibers, which help the phage attach to the host bacteria (e.g., T phages, see Figure 19-1). Other phages consist only of a capsid and nucleic acid and attach to the sex pili of male bacteria (male-specific bacteriophages, e.g., the MS-2 phage).

There are many potential applications of bacteriophages as environmental indicators (Table 19-1). These include their use as indicators of sewage contamination, efficiency of water and wastewater treatment, and survival of enteric viruses and bacteria in the environment. The use of bacteriophages as indicators of the presence and behavior of enteric bacteria and animal viruses has always been attractive because of the ease of detection and low cost associated with phage assays. In addition, they can be quantified in

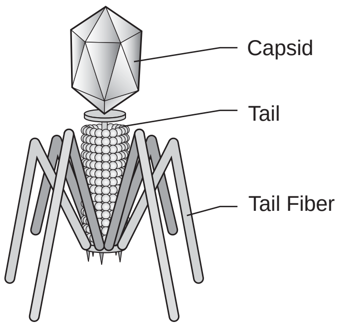


Figure 19-1 Coliphage T2.

Table 19-1 Potential applications of bacteriophage as environmental quality indicators

Detection of host organisms (i.e., fecal coliforms)
Water and wastewater treatment efficiency
Environmental fate of enteric viruses
Water movement in surface waters and groundwaters
Feces
Domestic sewage
Pathogens

environmental samples within 24 hours as compared to days or weeks for enteric viruses. Coliphages have been the most commonly used in this context although other bacteriophages and cyanophages (i.e., viruses of blue-green algae) have also been studied. Much of the justification for the study of coliphage behavior in nature has been to gain insight into the fate of human pathogenic enteric viruses. As a result, more is probably known about the ecology of coliphage than any other bacteriophage group.

Coliphages in water are assayed by addition of a sample to soft or overlay agar along with a culture of *E. coli* in the log phase of growth. The phage attach to the bacterial cell and lyse the bacteria. The bacteria produce a confluent lawn of growth except for areas where the phage has grown and lysed the bacteria. These resulting clear areas are known as plaques. A soft agar overlay is used to enhance the physical spread of the viruses between bacterial cells.

To obtain optimal plaque formation it is important that the host bacteria is in the log phase of growth. This ensures that all the phage attach to live bacteria and produce progeny. This requires that a culture of host bacteria be prepared each day that an assay is performed. Usually, a culture is incubated the day before the assay to obtain a culture in the stationary phase. This is then used to innoculate a broth which is incubated to obtain enough host bacteria in the log phase for the assay (this usually requires 2–3 hours of incubation in a shaking water bath at 35 to 37°C).

19.3. PROCEDURE

First Period

Materials

1.0 ml sewage (raw or non-disinfected treated sewage) or water sample containing coliphage
3–4 h nutrient broth culture of *E. coli*
2 tubes containing 9 ml of Tris-buffered saline (Tris buffer or other buffered saline)
4 tubes of 3 ml each of soft (top) agar (0.7% of nutrient agar or trypticase soy)
4 Petri dishes with bottom agar (10–12 ml) (nutrient agar or trypticase soy)
6 1-ml pipettes
pipette bulb
water bath at 45–48°C

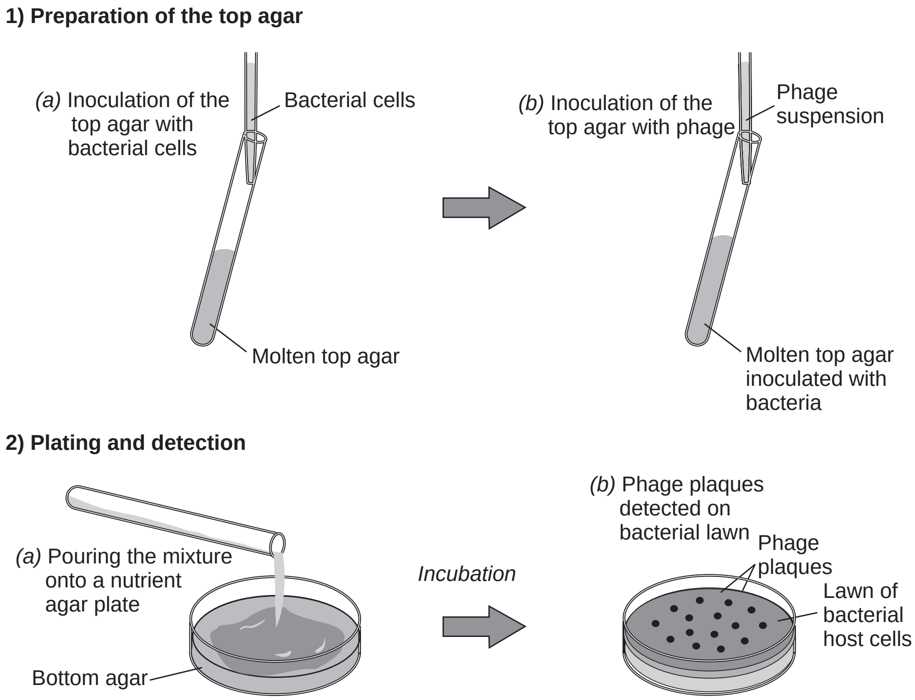


Figure 19-2 Procedure for the preparation of a bacterial lawn in the top layer of agar in which the detection of coliphage takes place.

paper towels
incubator at 37°C

1. A sample of sewage or water containing coliphage will be provided by the instructor.

2. Dilute the sample 1:10 and 1:100 by making 10-fold dilutions in Tris buffer by transferring 1.0 ml to 9 ml of Tris buffer.

3. Melt four tubes of soft agar (0.7% agar/3 ml tube) by placing in a steam bath or autoclaving. Place the agar in a water bath at 45–48°C and allow 15 min for the temperature of the agar to adjust to 45°C.

4. To the first tube add 1 ml of a log phase broth culture of *E. coli*[1] and 1 ml of undiluted sample. Remove the tube from the water bath and gently rock between your hands to mix the suspension for 2–3 seconds. Wipe the water from the tube with a paper towel and pour the agar over the Petri dish containing bottom agar. Quickly rotate the plate to spread the top agar. Be sure the agar covers the entire surface.

5. Repeat Step 4 using 1 ml of bacteria and 1 ml of each phage dilution. See Figure 19-2.

---

1. *E. coli* strain ATCC 15597 usually will produce the greatest number of plaques from sewage samples. A colony of *E. coli* ATCC 15597 is inoculated into 3 ml trypticase soy broth and incubated overnight at 35°C. Three hours before the phage assay inoculate 1 ml of this culture into a fresh flask containing 100 ml of nutrient or trypticase soy broth and place in a shaking waterbath at 35°C to 37°C. Incubate for 3 hours. This will ensure that the bacteria are in the log phase of growth.

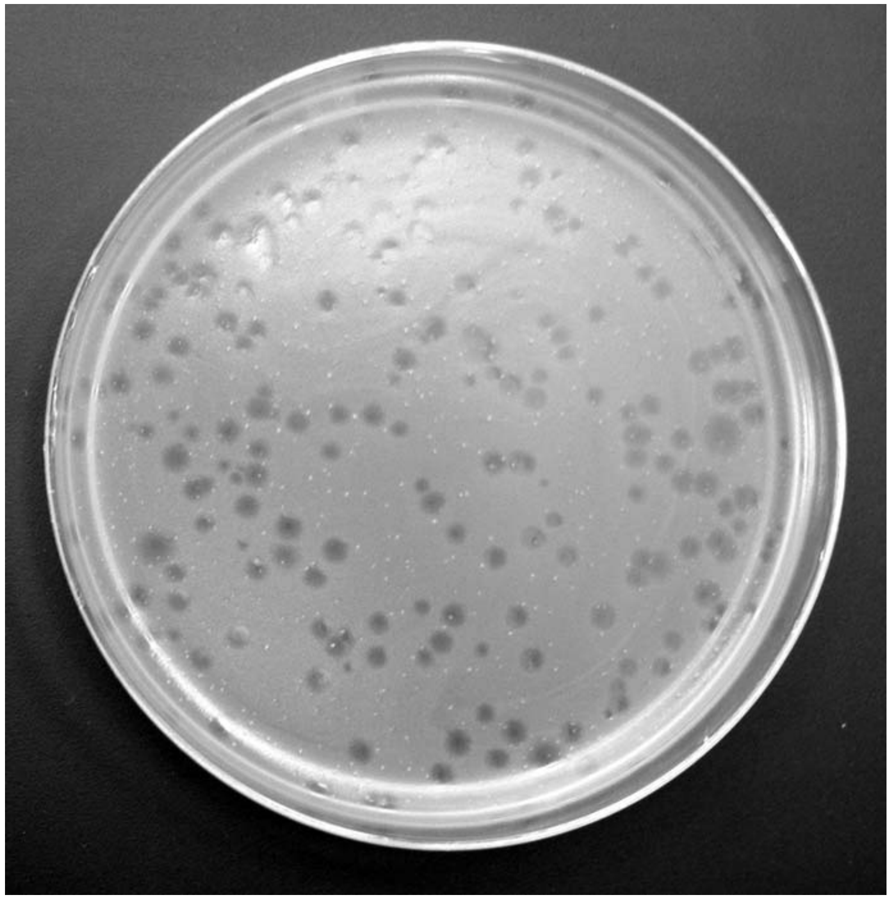


Figure 19-3 Phage plaques on a bacterial lawn. (Photo courtesy K.L. Josephson.)

6. After the agar has solidified, invert the Petri dishes and incubate at 37°C for 24 h. Knock any moisture off the lid of the Petri dish. If a drop of moisture falls on the plaque it will cause it to spread across the agar surface.

Second Period

Materials
incubated Petri dishes from Period 1

1. Count the number of plaques on each dilution (Figure 19-3) and calculate the concentration of phage in your original sample.

2. Record any major differences in the size or appearance of the plaques.

19.4. TRICKS OF THE TRADE

DO:

- Bacteria must be in the log phase of growth for optimal phage plaque formation. This means that a new culture must be grown under a

defined set of conditions (temperature, shaking or non-shaking) each time.

- Be sure to shake the tube containing overlay agar to get as much out of the tube as possible.

DO NOT:

- Do not allow the bacteria and phage to set in the water bath too long (no more than 1–2 minutes) or they will be killed by the heat.
- Do not allow the molten agar to set in the 45°C water bath for more than 1–2 hours as the water evaporates causing lumps of agar to form.

19.5. POTENTIAL HAZARDS

DO:

- Remember if you are handling sewage, it may contain pathogens. Handle with care.

19.6. QUESTIONS AND PROBLEMS

1. What are some factors that might determine plaque size?

2. Why do we use bacteria in the log phase of growth for this assay?

3. What are some sources of coliphage in the environment?

4. Why are coliphage potential indicators?

5. How is a plaque produced on a bacterial lawn?

19.7. REFERENCES

Bitton, G. (1998) *Wastewater Microbiology,* 2nd edition. Wiley-Liss, New York.

Goyal, S.M., Gerba, C.P., and Bitton, G. (1987) *Phage Ecology*. John Wiley & Sons, New York.

IAWPRC Study Group (1991) Bacteriophages as model viruses in water quality control. *Water Research* **5**, 529–546.

SECTION FIVE

Advanced Topics



Sampling apparatus to collect bioaerosols

Detection of Enteric Viruses in Water

EXPERIMENT **20**

20.1. OVERVIEW

Objective: ***To demonstrate how enteric viruses are concentrated and detected in water.***

- Demonstration of equipment used to concentrate viruses from water
- Observation of cell culture for viral cytopathogenic effects (CPE)

20.2. THEORY AND SIGNIFICANCE

Occurrence

Viruses excreted with feces from any species of animal may pollute water. Especially numerous, and of particular importance to health, are the viruses that infect the gastrointestinal tract of humans and are excreted with the feces of infected individuals. These viruses are transmitted frequently from person to person by the fecal-oral route. However, they also are present in domestic sewage which, after various degrees of treatment, is discharged to either surface waters or the land. Enteric viruses known to be excreted in relatively large numbers with feces include polioviruses, coxsackieviruses, echoviruses, and other enteroviruses, adenoviruses, reoviruses, rotaviruses, the hepatitis A (infectious hepatitis) virus, and the noroviruses. They are responsible for a wide range of diseases including gastroenteritis, skin rash, meningitis, myocarditis, eye infections, paralysis, fever, etc. With the possible exception of hepatitis A, each group or subgroup consists of a number of different serological types; thus more than 100 different human enteric viruses are recognized.

Viruses are not normal flora in the intestinal tract; they are excreted only by infected individuals, mostly infants and young children. Infection rates vary considerably from area to area, depending on sanitary and socioeconomic conditions. Because enteric viruses multiply only within living, susceptible cells, their numbers cannot increase in sewage. Sewage treatment, dilution, natural inactivation, and water treatment further reduce viral numbers. Large outbreaks of waterborne viral disease may occur when massive sewage contamination of a water supply takes place. It has been demonstrated that infection can be produced experimentally by ingestion of only a few virus units. Risk analysis has suggested that significant risk of infection could result from low numbers (one virus in 100 liters) of enteric viruses present in a drinking water supply.

Testing for Viruses

Detecting viruses in water through recovery of infectious virus requires three general steps: (a) collecting a representative sample; (b) concentrating the viruses in the sample; and (c) identifying and estimating quantities of the concentrated viruses. Particular problems associated with the detection of viruses of public health interest in the aquatic environment are: (a) the small size of virus particles (about 20–100 nm in diameter); (b) the low virus concentrations in water and the variability in amounts and types that may be present; (c) the inherent instability of viruses as biological entities; (d) the various dissolved and suspended materials in water and wastewater that interfere with virus detection procedures; and (e) the present limitations of virus estimation and identification methods.

Selection of Concentration Method

The densities of enteric viruses in water and wastewater usually are so low that virus concentration is necessary, except possibly for raw sewage in certain areas or seasons. Numerous methods for concentrating waterborne enteric viruses have been proposed, tested under laboratory conditions with experimental contaminated samples and, in some cases, used to detect viruses under field conditions.

Virus concentration methods often are capable of processing only limited volumes of water of a given quality. In selecting a virus concentration method consider the probable virus density, the volume limitations of the concentration method for that type of water, and the presence of interfering constituents. A sample volume less than one liter and possibly as small as a few milliliters may suffice for recovery of viruses from raw or primary treated sewage. For drinking water and other relatively nonpolluted waters, the virus levels are likely to be so low that hundreds or perhaps thousands of liters must be sampled to increase the probability of virus detection.

Currently, the method of choice for concentrating viruses from water is the adsorption/elution technique (APHA, 1998). This involves passing water through a filter to which the viruses adsorb and, subsequently, eluting (desorbing) the viruses off the filter using a one- or two-liter suspension of 1.5% beef extract.

Two types of filtering systems are used and both have advantages and disadvantages.

The electronegative filters have been shown to have a greater capacity for virus adsorption in waters with high turbidities and organic matter, but require the addition of $AlCl_3$ and acidification of the water to pH 3.5 to get the maximum adsorption of the viruses to the filter. This can be cumbersome as it requires modifying the water sample prior to filtering (addition of $AlCl_3$) and additional materials and equipment (pH meters). It also requires extensive training and experience for proper use.

Electropositive filters do not require any water preconditioning, but may clog more readily, and may not be as efficient for raw wastewater and other

waters high in organic matter. They cannot be used with waters with a pH above 8.5–9.0. A prefilter may also be used to increase the capacity of the virus filters but must be eluted and processed in the same manner as the virus adsorbing filters.

The most commonly used electronegative filter is the Filterite®. Generally it is used as a 10-inch (25.4 cm) pleated cartridge in either a 0.22 µm or 0.45 µm pore size rating (Gerba et al., 1978). The electropositive 1 MDS Virozorb® is especially manufactured for virus adsorption from water.

Once the viruses are eluted from the filters, they are concentrated (reconcentrated) to a smaller volume (usually 20–30 ml) before assay on animal cell culture. The entire procedure for concentrating viruses from water is shown in Figure 20-1.

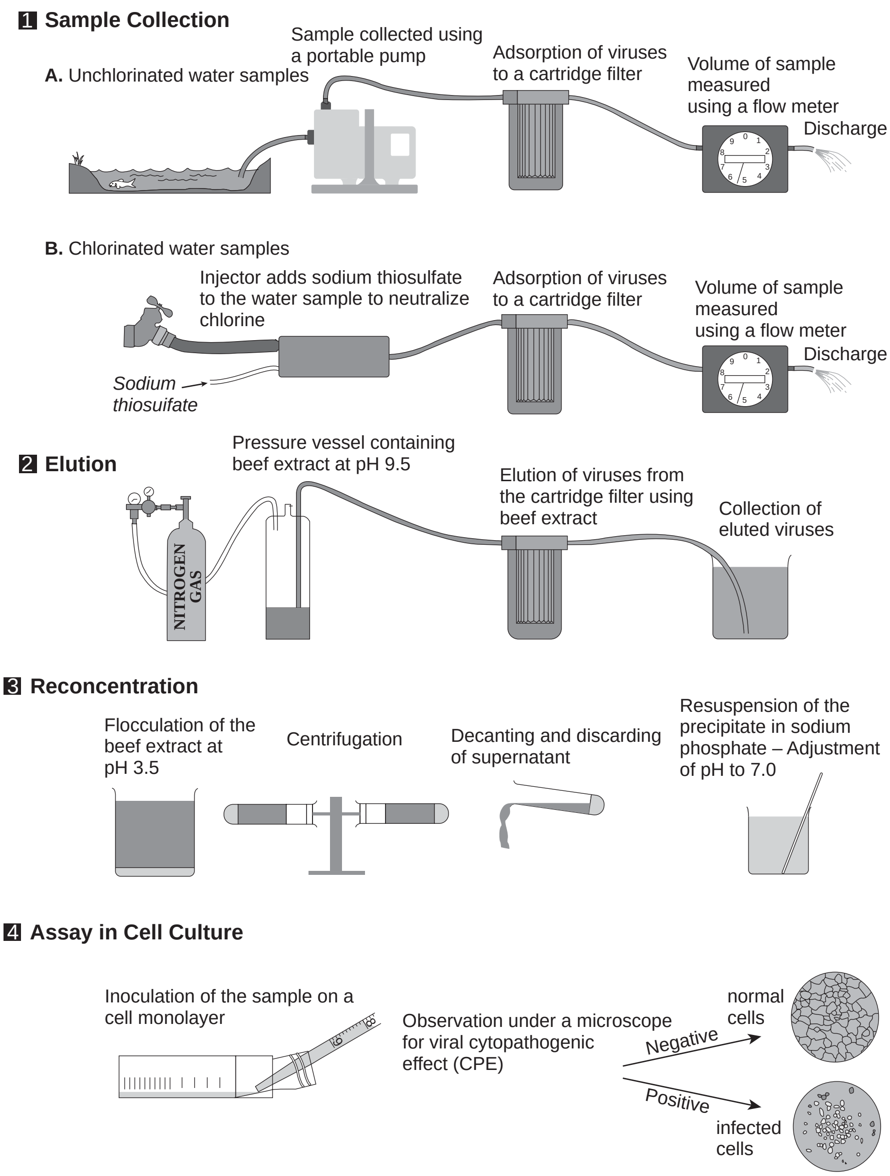


Figure 20-1 Procedure for concentration and detection of viruses in water.

Human enteric viruses are detected and quantified by their effects on monolayers of cells derived from human or animal tissues. Enteroviruses, reoviruses, and adenoviruses destroy the cells and display a cytopathogenic effect (CPE). Other viruses may grow in cells but do not cause CPE. Other viruses, such as Norwalk, cannot yet be grown in cell culture.

20.3. PROCEDURE

Materials

virus concentration equipment as depicted in Figure 20-1 (for demonstration purposes)

cell cultures infected with poliovirus type 1 (LSc—vaccine strain) in a culture flask

inverted light microscope

The equipment and procedures for enteric virus concentration from water will be demonstrated by the instructor. The basic procedures are as follows:

Collection and Filtering Procedures

1. Connect filter housing directly to a faucet or pump directly from water holding facility (steps 1A and 1B).

2. If it is necessary to dechlorinate, add sodium thiosulfate in-line (0.4 ml gal^{-1} (0.1 ml L^{-1}) of a 10% solution) either with the aid of an in-line injector or metering pump (step 1B). The water sample may also be collected in large plastic containers and dechlorinated as described for the electronegative filters.

3. Place the 1 MDS cartridge filter in the housing, secure and connect all tubing. The flow meter should be placed after the filter. A Filterite prefilter can be used if necessary (3-μm pore size), connected before the virus adsorbing filter.

4. Begin pumping a flow rate between 5–10 gal min^{-1} (19–38 L min^{-1}).

5. After the desired volume has been filtered, immediately place the filter at 4°C or on ice and ship to the laboratory for processing or elute the filter in the field. In contrast to electronegative filters, which must be shipped frozen, electropositive filters may be held at 4°C for up to 3 days before elution of adsorbed viruses (Sobsey and Glass, 1980).

Equipment Handling and Disinfection

6. Prior to use or shipment, the collection bottles and/or other plasticware should be autoclaved.

7. Non-autoclavable material such as hoses, filter housing, water meters, and pumps are exposed to 10 mg L^{-1} free chlorine for 30 min. The pH probes are disinfected by placement in 1 *M* HCl for 10 min.

8. After sampling, all equipment should be disinfected by exposure to 10–15 mg L^{-1} of chlorine (in the form of NaOCl) for 30 min and then

dechlorinated by the addition of sodium thiosulfate sufficient to neutralize the remaining free chlorine.

9. Flow meters should be placed downstream of filter housings (steps 1A and 1B).

Filter and Eluate Transportation

10. Filters can be left in the filter holder or can be placed in a plastic, resealable bag for shipping. Electropositive filters (1 MDS Virozorb®) must be shipped at 4°C to prevent significant die-off of the viruses. The electropositive filters should be eluted within 48–72 h after collection (Sobsey and Glass, 1980).

11. Filter eluates may be shipped or stored on ice at 4°C until received by the laboratory. They should be held no longer than 72 h at this temperature before freezing and storage at –1°C or lower. Samples may be frozen and shipped on dry ice. Frozen samples may be kept indefinitely.

Laboratory Procedures for Virus Isolation

When a water sample is brought into the laboratory, further processing of the virus sample is accomplished through elution, reconcentration, clarification, and assay on cell culture. Sometimes the elution step must be carried out in the field. Laboratory analysis may take anywhere from two to four weeks to complete. The following is a brief description of each step.

Filter Elution

12. Residual water is removed from the filter while in the holder.

13. One liter of 1.5% beef extract, with 0.05 M glycine at a pH of 9.5, is added to the filter holder and passed through the filter by applying air pressure. This elutes the virus off the filter (step 2, Figure 20-1).

14. The eluate is immediately adjusted to a neutral pH with 1 *M* HCl. The pH of eluate must be adjusted immediately after elution to prevent virus inactivation due to the high pH of the eluent.

Filter Eluate Reconcentration and Clarification

15. The one-liter eluate is reconcentrated by precipitation of the proteins and virus with acid followed by centrifugation (step 3, Figure 20-1).

16. The pellet from centrifugation is resuspended in 20–30 ml of buffer at a pH of 8–10.

17. The bacteria are removed through low speed centrifugation and treatment with antibiotics, if necessary.

18. The final sample is brought to a neutral pH.

Assay of Eluate on Cell Culture

19. The standard cell line used to assay environmental samples for enteroviruses is the Buffalo green monkey (BGM) kidney cell line. The cells are grown to confluent monolayers in plastic flasks (step 4, Figure 20-1).

20. The cells are examined for 14 days for cell destruction (CPE, cytopathogenic effect) caused by the virus. Any positive flask is confirmed by passages into fresh cells and subsequent CPE. At least half of the reconcentrated sample should be assayed.

21. Enteroviruses are usually quantified by the most probable number (MPN) method similar to coliform bacteria or by the plaque forming unit (PFU) method. In the PFU method, the cell monolayer is covered with agar. Then, the cell monolayer is exposed to a dye that only stains living cells. Cells killed by viruses produce clear zones or plaques in the monolayer.

Examination of Cell Culture

Your Assignment

22. Each student will examine cell cultures which have been infected with poliovirus type 1 LSc (the vaccine strain) under an inverted light microscope. Compare the infected cell monolayer to an uninfected cell monolayer. Record your observations.

20.4. QUESTIONS AND PROBLEMS

1. What type of diseases are caused by enteric viruses?
2. Why is it important to detect small numbers of enteric viruses in large volumes of water?
3. Why do viruses adsorb to electropositive filters?
4. What is CPE?
5. What is an enteric virus?
6. Why is beef extract used to concentrate viruses from water?

20.5. REFERENCES

APHA (1998) *Standard Methods for the Examination of Water and Wastewater.* 20th edition. American Public Health Association. Washington, DC.

Dahling, D.R., and Wright, B.A. (1984). Processing and transport of environmental virus samples. *Applied and Environmental Microbiology* **47**, 1272–1276.

Gerba, C.P., Farrah, S.R., Goyal, S.M., Wallis, C., and Melnick, J.L. (1978) Concentration of enteroviruses from large volumes of tap water, treated sewage, and seawater. *Applied and Environmental Microbiology* **35**, 540–548.

Katznelson, E., Fattal, B., and Hostovesky, T. (1976) Organic flocculation: and efficient second-step concentration method for the detection of viruses in tapwater. *Applied and Environmental Microbiology* **32**, 638–639.

Payment, P., and Trudel, M. (1981) Improved method for the use of proportioning injectors to condition large volumes of water for virological analysis. *Canadian Journal of Microbiology* **27**, 455–457.

Sobsey, M.D., and Glass, J.S. (1980) Poliovirus concentration from tap water with electropositive adsorbent filters. *Applied and Environmental Microbiology* **40**, 201–210.

Detection of Waterborne Protozoan Parasites

EXPERIMENT 21

21.1. OVERVIEW

Objective: ***Review how protozoan parasites are concentrated and detected in water.***

- Examine prepared slides of the free living and environmentally resistant stages of waterborne protozoan parasites.

21.2. THEORY AND SIGNIFICANCE

General

Enteric protozoan diseases are spread through the fecal-oral route, environmentally by the oocyst and cyst stages of *Cryptosporidium* and *Giardia*, respectively. They are spread both through drinking water and recreational contact (swimming pools) with surface waters.

There have been numerous waterborne outbreaks due to *Cryptosporidium* recorded in the world since 1987. The largest outbreak of cryptosporidiosis occurred in 1993 in the United States in Milwaukee, Wisconsin. The contamination of the lake and subsequent penetration of the oocysts through a drinking water treatment regime of coagulation, sedimentation, rapid sand filtration, and chlorination affected approximately 400,000 individuals.

Entamoeba histolytica is another protozoan parasite that has been associated with waterborne disease. However, while a common cause of gastroenteritis in the developing world, no waterborne outbreaks have been documented in the United States since 1953.

Microbiology of the Major Waterborne Parasites

Giardia

Giardia is a flagellated protozoan characterized by a simple life cycle involving two states—a cyst and a trophozoite. *Giardia* is shed in the feces of humans and animals, most often in a resistant cyst stage. Each viable, ingested cyst produces two flagellate trophozoites that attach themselves to the epithelial cells of the duodenum and jejunum. The cysts can survive in water for two months at 8°C, and are more resistant to chlorine than bacteria and *E. histolytica*. However, they are highly vulnerable to desiccation and

temperatures higher than 50°C. The cyst is football-shaped, averaging 8–12 μm in length by 7–10 μm in width. It contains inner structures, specific to *Giardia*, called median bodies and an axostyle which are used for the positive identification of cysts.

At present, since no routine methods exist for culturing *Giardia* cysts or *Cryptosporidium* oocysts from water samples, the only practical technique for detection in water relies on microscopically examining the concentrates derived from processing large volumes of water (10–400 liters). Thus, cyst recovery and positive identification depend on such factors as the amount of water pumped through the filter and the environmental factors affecting the distribution of the cyst in the aquatic environment. More importantly, cyst identification relies on the experience, skill, and persistence of the person analyzing the sample.

Cryptosporidium

Cryptosporidium is a coccidian protozoan parasite which develops within the gastric or intestinal mucosal epithelium in mammals. In contrast to *Giardia*, it has a relatively complicated life cycle involving sexual and asexual stages. It produces an environmentally stable oocyst which after ingestion undergoes excystation, releasing 4 sporozoites which then initiate an intracellular infection within the epithelial cells of the gastrointestinal tract. The sporozoite differentiates into a trophozoite which undergoes asexual multiplication to form type I meronts and then merozoites which may infect new cells. Merozoites from type II meronts produce microametocytes and macrogametocytes which undergo sexual reproduction to form the oocyst. The oocysts are then excreted into the feces. The oocysts are very resistant to common disinfectants, even more so than *Giardia*. They are smaller than *Giardia*, being from 4 to 6 μm in diameter and round in shape. Cattle and other animals may serve as reservoirs of *Cryptosporidium*.

Entamoeba Histolytica

The life cycle of *E. histolytica* is characterized by three stages: trophozoite, precyst, and cyst—the cyst being the infective stage. The cyst stage is resistant, but it cannot withstand temperatures above 50°C, sunlight, or extended exposure to disinfectants. All three stages of the organism may be found in the stools of infected persons. Unlike *Giardia* and *Cryptosporidium*, humans and subhuman primates are the only reservoirs of *E. histolytica*.

Aspects of the microbiology of the major waterborne protozoan parasites occurring are compared in Table 21-1.

Methods of Detection in Water

Like enteric viruses, only a few protozoan parasites need be ingested to cause infection in man. Therefore, large volumes of water (10–400 liters) are sampled. The cysts or oocysts are collected by passing the water being tested through a pleated cartridge filter. The volume of water pumped through the filter depends on the purpose of the investigation. After sampling is

Table 21-1 Comparison of major waterborne Protozoa

	Cryptosporidium	*Entamoeba*	*Giardia*
Source of environmental contamination	Infected humans and animals	Infected humans and chronic carriers	Infected humans and animals, chronic infections
First documentation of waterborne disease	1986	1929	1965
Stage present in water	Oocyst	Cyst	Cyst
Major cause of waterborne disease	Filtered and disinfected water supplies	Sewage contamination of potable waters	Use of unfiltered surface waters as drinking water

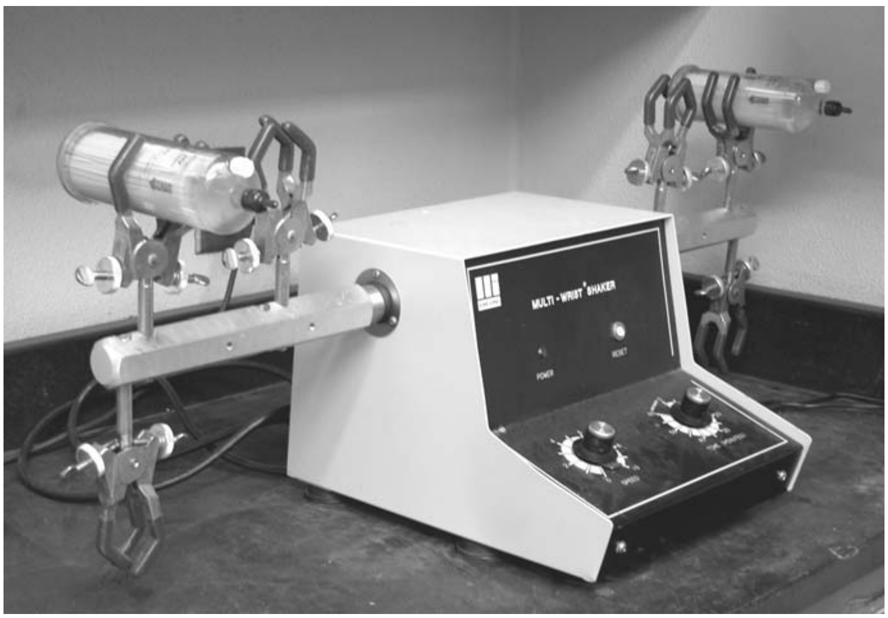


Figure 21-1 Apparatus used for recovery of cysts or oocysts from the pleated filter. (Photo courtesy K.L. Josephson.)

completed, the filter cartridge is placed in a Ziplock®[1] bag, double-bagged, sealed, labeled, placed on ice, and processed within 48 h.

Cysts and oocysts are stable for many weeks at 4°C when stored in formalin or potassium dichromate. Recovery of the cysts or oocysts involves the use of an elution buffer and shaking which aid their detachment from the filter. In the laboratory, elution buffer is added to the inlet end of the capsule filter ensuring that the pleated filter is covered with solution. The filter capsule is attached to a shaker and shaken for 5 minutes at 600 rpm (Figure 21-1). The solution is decanted into a 250-ml conical centrifuge tube. Elution buffer is again added to the filter with an additional 5-minute shaking period. This solution is also decanted into a 250-ml conical centrifuge tube. The solution is centrifuged at 1000 × g for 10–15 minutes to pellet the cysts and oocysts

1 Dow Brands L.P., Indianapolis, Indiana.

and the supernatant is aspirated to the pellet. The pellet volume is measured and recorded, and adjusted to bring the final volume up to 10 ml (if pellet is less than 0.5 ml), or the equation below can be used if the pellet volume is greater than 0.5 ml:

$$\text{total volume required (ml)} = \frac{\text{pellet volume} \times 10\,\text{ml}}{0.5\,\text{ml}}$$

Immunomagnetic Separation

Algae and other particulate suspended matter (clays, soil) are also concentrated along with the oocysts and cysts which can interfere with their observation under the microscope. To separate the oocysts/cysts, immunomagnetic separation is used. This involves the addition of magnetic beads which are coated with specific antibodies to the cysts and oocysts.

After attachment of the cysts/oocysts to the beads, the beads are separated from solution with the use of a magnet. The cysts/oocysts are then placed in a solution to disassociate them from the magnetic beads.

1 ml of 10X SL-buffer-A and 1 ml of 10X SL-buffer-B (supplied in Dynal IMS kit[1]) is added to a flat-sided sample tube; 10 ml of the water sample concentrate is then added to the flat-sided sample tube along with 100 µl of Dynabeads Crypto-Combo and 100 µl of Dynabeads Giardia-Combo magnetic beads from the Dynal IMS kit. The sample tube is attached to a rotating mixer and rotated at 18 rpm for 1 hour. The sample tube is placed in the MPC-1 (magnetic particle concentrator) with the flat side of the tube next to the magnet. The supernatant is poured out of the tube. The sample is resuspended in 1 ml of 1X SL-buffer-A and placed in a second magnetic particle concentrator (MPC-M); the supernatant is aspirated from the tube. The magnetic strip is removed from the MPC-M and 50 µl of 0.1 N HCl is added to the sample tube and vortexed for 10–15 seconds. After 10 minutes, the tube is placed in the MPC-M, the supernatant is removed and placed on the first well containing 5 µl of 1.0 N NaOH on a well slide. The sample is air-dried. This procedure is repeated twice to ensure the organism has disassociated from the magnetic bead and the supernatant is placed in the second well of the slide (~50 µl/well)

Staining

To aid in the detection of *Giardia* cysts and *Cryptosporidium* oocysts, direct or indirect immunofluorescent microscopy using monoclonal antibodies is often used when examining environmental concentrates. Fluorescein isothiocyanate (FITC) is conjugated to the antibodies and epifluorescent microscopy used for final detection. A characteristic apple-green fluorescence around the cyst or oocyst is seen.

A positive and negative control well should be prepared each time the staining is performed. One drop of Detection Reagent and one drop of

---

1 Dynal Biotech Inc., Brown Deer, Wisconsin.

Table 21-2 Procedures for the determination of protozoan parasites in water samples.

Sample Step	Procedures Used
Sample Collection	Ten to 100 L of water passed through a pleated cartridge membrane filter
Elution and Recovery	Elution buffer added to inlet end of capsule filter and shaken for 5 minutes at 600 rpm
Concentration and Clarification	Centrifugation is used to concentrate the cysts and oocysts from the eluting media. The pellets are magnetized by attachment of magnetic beads conjugated with antibodies against *Giardia* and *Cryptosporidium*. The magnetized cysts and oocysts are separated from the solution using a magnet. The magnetic bead complex is then disassociated from the cysts and oocysts using a weak acid solution.
Detection	The supernatant is placed in a well slide and stained with specific antibodies directed against the cyst or oocyst in a direct fluorescent procedure.

Counterstain Reagent is added to each well that contains sample. The slide is incubated in a humid chamber in the dark at room temperature for ~30 minutes. After this time, 1 drop of wash buffer is added to each well and gently aspirated from below the well using a clean Pasteur pipette without disturbing the sample. One drop of mounting medium is added to each well, a coverslip is applied and sealed with fingernail polish and enumerated by fluorescent microscopy.

A summary of the methods used in investigating water samples for waterborne parasites is given in Table 21-2.

21.3. PROCEDURE

Materials

prepared slides[1] of the following:
- *Giardia lamblia* cysts
- *Giardia lamblia* trophozoites
- *Entamoeba histolytica* trophozoites
- *Cryptosporidium* oocysts

microscope

You will be given a series of prepared slides to examine. Draw any observations of the following: *Giardia lamblia* cysts, *Giardia lamblia* trophozoites, *Entamoeba histolytica* trophozoites, *Cryptosporidium* oocysts. Note the size and shape of the organisms. Note and draw any internal features which you observe.

21.4. QUESTIONS AND PROBLEMS

1. Which of the parasites only infects primates?

2. Which of the parasites produces an oocyst?

1 Slides may be obtained from North Carolina Biological Supply.

3. What drinking water treatment process is most effective in removing parasite cysts and oocysts from water?

4. What is the infective stage of *Giardia* found in water called?

5. Why is it necessary to sample large volumes of water for cysts and oocysts?

6. How are the cysts/oocysts removed from the filter used to concentrate them from water.

7. What is the purpose of the magnetic beads used to detect the cysts/oocysts?

8. What is the purpose of fluorescein isothiocyanate labeled antibodies?

9. Which is larger, *Giardia* cysts or *Cryptosporidium* oocysts?

21.5. REFERENCES

Rose, J.B. (1990) Occurrence and control of *Cryptosporidium* in drinking water. *Drinking Water Microbiology*. G.A. McFeters, ed., pp. 298–321. Springer-Verlag, New York.

Rose, J.B., Gerba, C.P., and Jakubowski, W. (1991) Survey of potable water supplies for *Cryptosporidium* and *Giardia*. *Environmental Science and Technology* **25**, 1393–1400.

United States Environmental Protection Agency (2001) Method 1623: *Cryptosporidium* and *Giardia* by Filtration/IMS/FA. Office of Water. Washington, DC.

Kinetics of Disinfection

EXPERIMENT 22

22.1. OVERVIEW

Objective: ***To determine the rate of bacterial inactivation by ultraviolet light disinfection.***

- Expose *Escherichia coli* to UV light for different periods of time
- Assay exposed samples after dilution by the spread plate method
- Incubate samples overnight
- Count bacterial colonies and determine concentration after different exposure times to the UV light
- Plot data on semi-log paper and determine the dose for 99% reduction of the *E. coli*

22.2. THEORY AND SIGNIFICANCE

Disinfection of water and wastewater is important in the control of waterborne disease. Chlorine, chlorine dioxide, ozone, and ultraviolet light are common disinfectants used in the drinking and wastewater industries. They are also used to kill or inactivate microorganisms on inanimate (called fomites) surfaces and foodstuffs. Inactivation of microorganisms is a gradual process that involves a number of physical–chemical and biochemical processes. The speed of these processes is dependent on a number of factors, the most important being the dose or concentration of the disinfectant, temperature, pH, the concentration of suspended and organic matter in the water, and the presence and concentration of dissolved salts. It is important to predict the rate of inactivation of microorganisms so that the proper amount of disinfectant is applied to achieve the desired amount of inactivation. For example, the United States Environmental Protection Agency requires that drinking water treatment plants which treat surface waters be capable of removing and/or inactivating enteric viruses by 99.99%. To accomplish this it is necessary to be able to predict the amount of microbial inactivation under a given set of water quality conditions. In the case of chemical disinfectants the disinfectant effectiveness is expressed as *Ct*, where *C* is the disinfectant concentration and *t* the time required to inactivate a certain percentage of the population under a specific set of conditions (pH and temperature). Typically, a level of 99% or 99.9% inactivation is used when comparing *Ct* values. In general the lower the *Ct* value, the more effective the disinfectant. Protozoan cysts and oocysts are the most resistant to oxidizing disinfectants (e.g., chlorine, chlorine dioxide, ozone) followed by viruses and then bacteria.

The use of ultraviolet disinfection of water and wastewater has seen increased popularity in recent years because it is not known to produce toxic disinfectant by-products, which are produced with the use of chlorine and

ozone. It also has the advantage that pH and the temperature of the water do not affect its effectiveness. However, there are higher costs associated with the use of UV light, no disinfectant residual is left in the water, and the water must be of low turbidity. UV light inactivates microorganisms by damaging microbial DNA or RNA at a wavelength of 260 nm. It causes thymine dimerization, which blocks nucleic acid replication and effectively inactivates microorganisms. Microbial inactivation is proportional to the UV dose, which is expressed in microwatt-seconds per square centimeter ($\mu W\text{-}s/cm^2$) or

$$\text{UV dose} = It$$

where $I = \mu W/cm^2$ and t = exposure time. In most disinfection studies, it has been observed that the logarithm of the surviving fraction of organisms is nearly linear when plotted against dose. A further observation is that constant dose yields constant inactivation. This can be expressed as:

$$\text{Log}\,\frac{N_s}{N_i} = \text{function } (It)$$

where N_s is the density of surviving organisms (number/cm^3) and N_i is the initial density of organisms before exposure (number/cm^3). Because of the logarithmic relationship of microbial inactivation versus UV light dose, it is common to describe inactivation in terms of log survival or

$$\text{log survival} = \log\frac{N_s}{N_i}$$

For example, if one organism in 1000 survived exposure to UV light, the result would be a 3 log reduction. Determining the UV susceptibility of various indicator pathogenic microorganisms is fundamental in quantifying the UV dose required for adequate water disinfection.

UV inactivation data is usually collected by placement of a suspension of organisms in a stirred, flat, thin-layer dish. In UV batch reactors there are uniform UV intensities and contact time can be controlled.

Viruses are the most resistant to inactivation by UV light followed by bacteria and then protozoan cysts and oocysts.

22.3. PROCEDURE

First Period

Materials

UV light source
100 ml of phosphate buffered saline
1 50 × 12 mm glass or plastic Petri dish
1 7 × 2 mm stir bar
1 ring stand
1 clamp for ring stand

1 ruler
12 nutrient of trypticase soy agar plates
4 81-ml pipettes
4 10-ml pipettes
glass hockey stick for spread plating
gas burner
12 dilution tubes containing 9 ml of saline
vortex mixer
timer
test tube rack
goggles to prevent eye UV light exposure
stir plate

Overnight grown culture of *Escherichia coli*

1. Set up UV light source and stir plate as shown.

2. Turn on the UV light 10 minutes before you leave and keep on during the course of the experiment.

3. Obtain a suspension from the instructor of approximately 10^5 *Escherichia coli* per ml.

4. Place a suspension of 10 ml of an *E. coli* (10^5 organisms per ml) in a normal saline solution with a 10-ml pipette.

5. Put on a pair of UV protective goggles during exposure of the bacteria to the UV light source.

6. Place a 1 mm stir bar in the Petri dish and place under the UV light source.

7. Expose the bacteria in the Petri dish to the UV light for 30 seconds.

8. Remove the Petri dish from under the UV light source and conduct a 10^{-1}, 10^{-2}, 10^{-3} dilution, and assay by the spread plate method as described in Experiment 4.

9. Repeat Steps 4 thru 8 but increase exposure to 60 seconds, 90 seconds, and 120 seconds.

10. Incubate the agar plates for 24 hours at 35°C.

Second Period

Materials
incubated plates from Period 1
semi-log paper

1. Count the number of bacterial colonies on each dilution.

2. Calculate the number of bacteria per ml for each of the four samples.

Table 22-1 UV dose to kill enteric microorganisms

Organism	**Ultraviolet dose (μW-s/cm^2) required for 90% reduction**
Escherichia coli	1,300–3,000
Salmonella typhi	2,100–2,500
Shigella dysenteriae	890–2,500
Adenovirus	23,600–30,000
Poliovirus	5,000–12,000
Hepatitis A	3,700–7,300
Coliphage MS2	18,600
Cryptosporidum	2,700–6,700

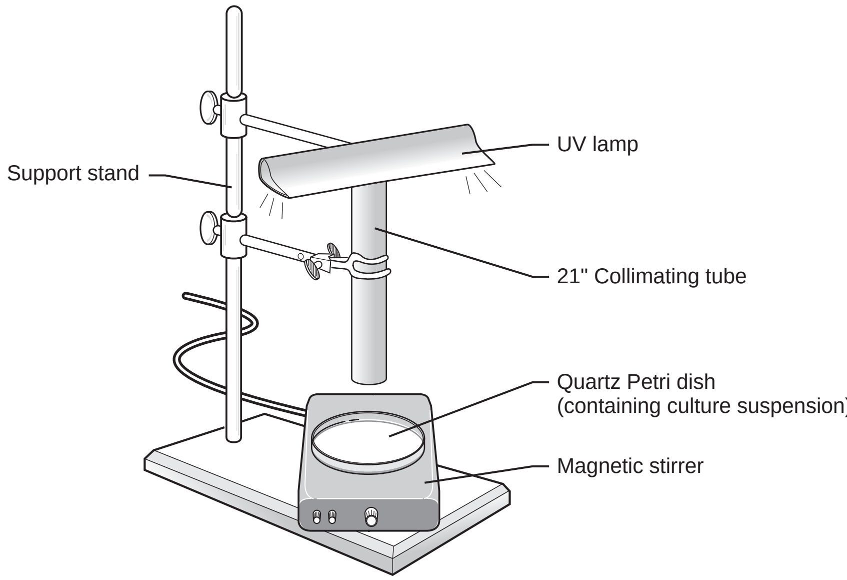


Figure 22-1 Collimating tube apparatus for UV dose application.

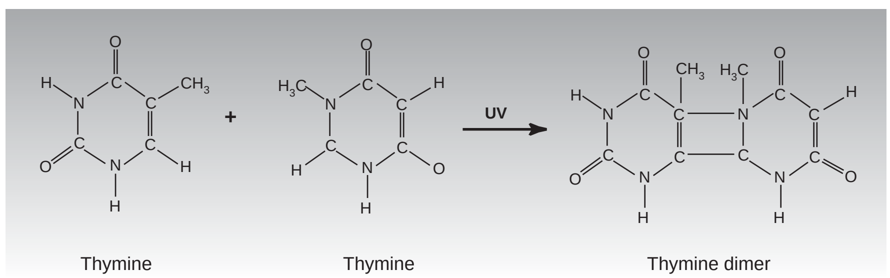


Figure 22-2 Formation of thymine dimers in the DNA of irradiated nonsporulating bacteria.

3. Graph the concentration of bacteria per ml for each exposure vs. exposure time. You should obtain a straight line.

4. Determine the time needed for 99% inactivation of *Escherichia coli*.

22.4. TRICKS OF THE TRADE

DO:

- Place the Petri dish used to expose the bacteria to the UV in the same place each time. Mark the spot with marker circle so the plate is in the same place each time.

DO NOT:

- Change the distance of the UV light source from the Petri dish as this changes the UV light dose.

22.5. POTENTIAL HAZARDS

<u>DO</u>:

- Looking directly at the UV light source can cause damage to your eyes. Always wear goggles when working with UV light sources.

22.6. CALCULATIONS

None

22.7. QUESTIONS AND PROBLEMS

1. How does UV light inactivate bacteria and viruses?

2. Why are viruses more resistant to the inactivation of UV light than vegetative bacterial cells?

3. If a dose of 8000 μW/cm^2 is needed to inactivate 90% of the poliovirus in a water sample what dose is needed to inactivate 99.9% of this virus?

4. What is *Ct*? How is it used in the drinking water industry?

5. What is *It*? How is it used in the water industry?

6. What is an advantage of using UV light disinfection instead of chlorine disinfection of drinking water? What is a disadvantage?

22.8. REFERENCES

Maier, R., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology.* Academic Press, San Diego, CA.

Roessler, P.F., and Severin, B.L. (1996) Ultraviolet disinfection of water and wastewater. In: *Modeling Disease Transmission and its Prevention by Disinfection*. C.J. Hurst, ed., pp. 313–368. Cambridge University Press, UK.

The same UV light source used in Experiment 19. The UV light sources and goggles can be obtained from IDEXX Laboratories, Westbrook, ME. www.idexx.com

50 mm Petri dishes can be obtained from the Millipore Corporation. Bradford, MA. www.millipore.com or most laboratory supply companies.

Aerobiology: Sampling of Airborne Microorganisms

EXPERIMENT 23

23.1. OVERVIEW

Objective: ***To collect air samples for subsequent microbial analysis.***

- Collect bioaerosol samples with a glass impinger and filtration apparatus
- Assay samples for airborne bacteria and fungi by the spread plate and membrane filtration methods
- Determine the concentration of bacteria and fungi per cubic meter of air

23.2. THEORY AND SIGNIFICANCE

Aerobiology has been defined as the study of aerosolization, aerial transmission, and deposition of biological materials. A collection of airborne biological particles is called a bioaerosol. Bioaerosols are generated by a wide variety of natural and human-made processes including coughing, sneezing, wave action, splashes, wind, cooling towers, ventilation systems, etc. Inhalation, ingestion, and dermal contact are routes of human exposure to airborne microorganisms, but inhalation is the predominant route that results in adverse human health effects. Airborne *Legionella pneumophila, Mycobacterium tuberculosis*, and some pathogenic viruses are known to be transmitted by aerosols. Asthma, hypersensitivity pneumonitis and other respiratory illnesses are also associated with exposure to bioaerosols.

Deterioration of building materials, offensive odors, and adverse human health effects are associated with microbial contamination of indoor environments, such as residences, offices, schools, health care facilities, enclosed agricultural structures (barns and crop storage areas) industrial facilities and recycling facilities (Stetzenbach, 2003). Sources and reservoirs of microorganisms are present within these settings, including building materials and furnishings, pets, plants, and air-conditioning systems. Fungi, which can colonize drier surfaces than bacteria, tend to grow in a wide variety of building materials, such as wallboard, ceiling tiles, carpeting and vinyl flooring.

Temperature and relative humidity are the two most important factors affecting the survival of microorganisms in the airborne state. For this reason these are usually recorded during the collection of bioaerosols. In general, bacteria and fungi are more stressed as the rate of evaporation increases, which occurs as relative humidity deceases and temperature increases. Thus,



Figure 23-1 37 mm polystyrene 3-piece monitoring cassette. (Photo courtesy Millipore Corporation.)

increased survival is favored at higher relative humidity and lower temperatures. The effect of relative humidity varies more with virus type with some surviving better at high relative humidity and others better at low relative humidity.

There are three principal methods used to quantify microorganisms in the air.

- **Impingement** is the trapping of airborne practices in a liquid matrix
- **Impaction** is the forced deposition of airborne particles on a solid surface
- **Filtration** is the trapping of airborne particles by size exclusion

Gravity is a non-quantitative method used in which agar medium is exposed to the environment and airborne microorganisms are collected primarily by settling. This method is often used because it is inexpensive and easily performed.

A commonly used liquid impinger is the AGI-30 (ACE Glass, Vineland, NJ). The AGI-30 operates by drawing air through the inlet and into a liquid. Any particles in the air become trapped in the liquid, which can then be assayed for the presence of microorganisms. The AGI-30 is usually operated at a flow rate of 12.5 liters per minutes. The AGI-30 is easy to use, inexpensive, portable, reliable, easily sterilized, and has high biological sampling efficiency in comparison with many other sampling devices. The usual collection volume is 20 ml, and the typical sampling time is about 20 minutes. Longer sampling times result in too much evaporation of the liquid in the impinger, and the inactivation or death of microorganisms in the liquid.

The impaction method separates particles from the air by utilizing the inertia of the particles to force their deposition onto a solid or semi-solid surface. The collection surface is usually an agar medium. The Anderson six-stage impact or sampler (Anderson Instruments Inc., Smyra, GA) consist of six stages with decreasing nozzle diameters, so that successive stages collect progressively smaller particles. Thus the six-stage sampler measures the cultivable bioaerosol concentrations in specific particle size ranges.

Filtration techniques are used largely for the collection of fungi and bacterial spores because they are desiccation resistant. Filters are usually held in disposable (although they may be reused) plastic filter cassettes during bioaerosol sampling. Membrane filters used for sampling are usually supplied as disks of 37- or 47 mm diameter. Because the pressure drop across a filter increases with air velocity through the filter, the use of larger filters results in a lower pressure drop for a given volumetric flow rate. The use of the smaller (37-mm) filter concentrates the organisms onto a smaller total area, thus increasing the density of particles per unit area of the filter. This may be helpful for direct microscopic examination of low concentrations of organisms. In areas of high concentration, the organisms may have to be eluted, diluted, and then refiltered for microscopic examination or assay. For a better quantitative measure of the air volume sampled, a limiting orifice may be placed between the cassette and the vacuum source.

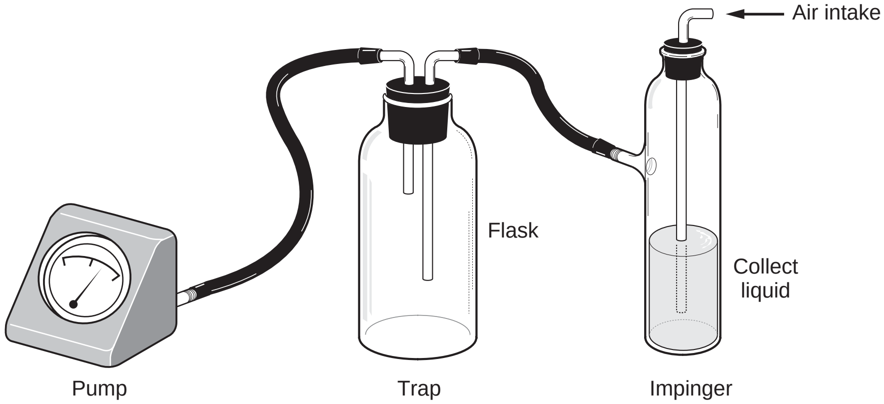


Figure 23-2 Liquid impingement device for collecting biological aerosols.

23.3. PROCEDURE

First Period

Materials

All glass impinger AGI-30
1 37-mm air monitoring cassette
20 ml of 0.1% peptone solution
1 500- or 1000-ml Erlenmeyer flask
rubber or plastic tubing for connecting the impinger and cassette to the vacuum source
1 vacuum pump or vacuum source
1 100 ml sterile graduated cylinder
1 dilution blank with 0.1% peptone or phosphate buffered saline
2 1-ml pipettes
1 10-ml pipette
4 sterile 0.45 µ pore, 47-mm diameter membrane filters
1 filter unit (same as in Experiment 10)
2 sterile 37 mm 0.45 µm pore filters
vacuum pump or other source
forceps
gas burner
pipette bulb
vortex mixer
4 nutrient agar (NA)[1] or trypticase soy agar (TSA)[1] plates
4 Sabouraud dextrose agar (SDA)[1] plates

Air Sampling by Impingement

1. Set up the AGI-30 all glass impinger as shown (Figure 23-2).

2. Add 20 ml of 0.1% peptone to the reservoir followed by 0.1 ml of anti-foam.

1 Anti-foam B (Sigma Chemical Company, St. Louis, Missouri).

3. Add 0.1 ml of anti-foam agent.

4. Turn the vacuum source on for 10 minutes.

5. With a 1-ml pipette remove 0.5 ml of fluid from the reservoir and place 0.1 ml each on one agar plate of either NA or TSA and spread plate the samples as described in Experiment 5. Place another 0.1 ml on a plate of Sabouraud dextrose agar for detection of fungi.

6. With a 10 ml pipette remove 6 ml of liquid from the reservoir and pass 5 ml through a 0.45 μm membrane filter as described in Experiment 10. Place the filter on an NA or TSA plate. Repeat the procedure, but place the membrane filter on Sabouraud dextrose agar.

7. Incubate the NA or TSA plates at 35°C for 24–48 hours.

8. Incubate the SDA for 2 to 7 days.

Air Sampling by Filtration

9. Connect the air-sampling cassette to the vacuum source.

10. Turn on the vacuum source for 10 minutes.

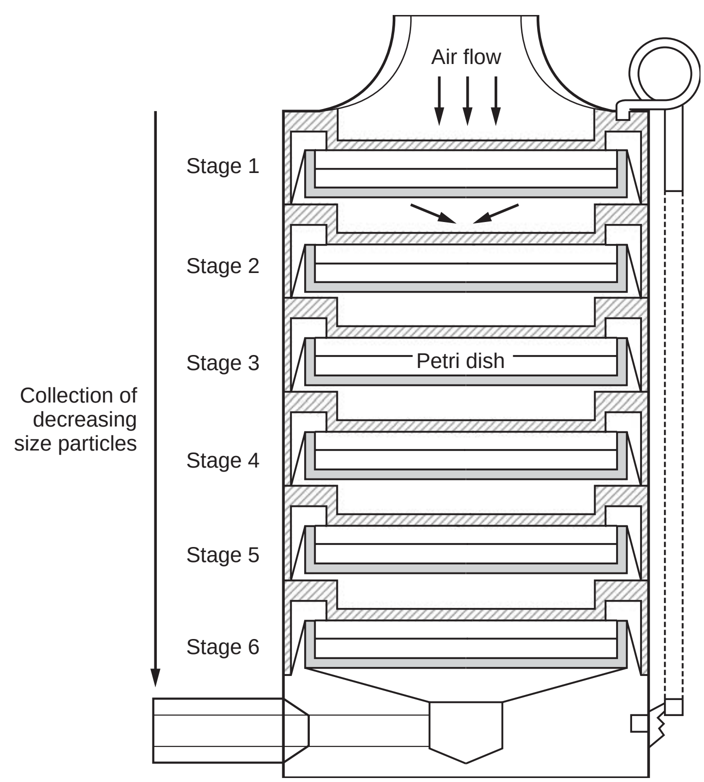


Figure 23-3 Sieve-type sampler (Andersen sampler) for biological aerosols.

11. Remove the membrane filter from the cassette with a pair of flamed forceps (see Experiment 10). And place on either a plate of NA or TSA.

12. Repeat the same procedure placing the membrane filter on plate of SDA.

13. Incubate the plates as described under impingement.

Second Period

Materials
incubated plates from the previous Period 1

1. Examine the agar plates and count the umber of bacteria (NA or TSA) and fungi (SDA) colonies.

23.4. TRICKS OF THE TRADE

DO NOT:

- Leave the vacuum on for more than 10 minutes as the fluid in the AGI-30 will decrease significantly in volume and the bacteria in the filtration cassettes will desiccate.

23.5. CALCULATIONS

Calculate the number of bacteria and fungi per cubic meter. The AGI-30 limiting orifice at the end of the glass tube, which is submerged into the collection liquid, limits the amount of air passing through the liquid to 12.5 liters per minute. The concentration of microorganisms is usually reported as numbers per cubic meter of air, which is calculated as follows.

A) Volume of air (L) = Sample time (min) × 12.5 L/min

B) $$\text{Number of organisms collected by impinger} = \text{Number of organisms in volume assayed (CFU/ml)} \times \text{ml remaining in impinger after operation*}$$

C) $$\text{Number of organisms per L of air (CFU)} = \frac{\text{Number of organisms collected by impinger}}{\text{Volume of air}}$$

23.6. QUESTIONS AND PROBLEMS

1. What are the limitations of the different methods for detection of microorganisms in air?

*Remember the volume of the liquid in the impinger may decrease during operation because of evaporation.

2. Why should you not collect an air sample for more than 20 minutes with an AGI-30?

3. Why are fungi of concern in the air?

4. Why are bacterial spores and fungi more likely to be detected with a filtration cassette than vegetative bacterial cells?

5. What types of environments would you expect to have high concentrations of bacteria in the air? Low concentrations?

6. How does relative humidity effect the survival of airborne microorganisms?

23.7. REFERENCES

Buttner, M.P., Willeke, K., and Grinshpun, S.A. (2003) Sampling and analysis of airborne microorganisms. In: *Manual of Environmental Microbiology*. C.J. Hurst, R.L. Crawford, G.R. Knudsen, M.J. McInerney, and L.D. Stetzenbach, eds., pp. 814–826. ASM Press. Washington, DC.

Dowd, S.C., and Maier, R.M. (2000) Aerobiology. In: *Environmental Microbiology*. R.M. Maier, I.L. Pepper, and C.P. Gerba, eds. pp. 91–122. Academic Press. San Diego, CA.

Durand, K.H., Muilenberg, M.L., Burge, H.A., and Seixas, N.S. (2002) Effect of sampling time on the culturability of airborne fungi and bacteria sampled by filtration. *American Occupational Hygiene* **46**, 113–118.

Lundholm, I.M. (1982) Comparison of methods for quantitative determination of airborne bacteria and evaluation of total viable counts. *Applied and Environmental Microbiology* **44**, 179–183.

Stetzenbach, L.D. (2003) Introduction to Aerobiology. In: *Manual of Environmental Microbiology*. C.J. Hurst, R.L. Crawford, G.R. Knudsen, M.J. McInerney, and L.D. Stetzenbach, eds., pp. 801–813. ASM Press. Washington, DC.

AGI-30 can be obtained from Ace Glass Inc., Vineland, NJ. www.aceglass.com.

Air sampling cassettes, 37 mm filters, and vacuum pumps can be obtained from Pall Gelman Laboratory, Ann Arbor, MI. www.pall.com/gelman and Millipore Corporation, Bedford, MA. www.millipore.com.

EXPERIMENT 24

Detection and Identification of Bacteria Via PCR and Subsequent BLAST Analysis of Amplified Sequences

24.1. OVERVIEW

Objective: ***To introduce students to the powerful technology known as polymerase chain reaction (PCR) which allows for DNA within specific bacteria to be amplified and analyzed. Analysis of amplified sequences allows for detection and identification of the target bacteria.***

- Amplify 16S rRNA bacterial sequences via polymerase chain reaction (PCR)
- Verify amplification by gel electrophoresis and ethidium bromide staining
- Sequence the 16S rRNA fragment and identify bacteria via Basic Local Alignment Search Tool (BLAST) analysis

24.2. THEORY AND SIGNIFICANCE

Nucleic acids consist of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). DNA is made up of four deoxynucleotide bases: guanine (G), cytosine (C), adenine (A), and thymine (T). Guanine and adenine are purine bases, whereas cytosine and thymine are pyrimidine bases. DNA consists of these bases linked to the sugar deoxyribose and a phosphate moiety. Structurally, DNA consists of two strands of these bases combined together to form a double helix. One strand of DNA is oriented 5′ to 3′ while the complimentary strand is oriented 3′ to 5′. These two strands are linked by hydrogen bonds between corresponding pairs of bases. Specifically G binds only to C and A only binds to T. Thus if the sequence of one strand is known, the sequence of the complimentary strand can be deduced. The double-stranded complimentary nature of DNA is the basis for the methodology known as polymerase chain reaction or PCR. PCR can be used to amplify bacterial rRNA allowing for specific detection and identification at the species level and in some cases to the strain or biovar level. PCR can also be used to amplify RNA associated with certain viruses. RNA is similar to DNA, consisting of a single strand of A, G, and C bases, but uracil (U) substitutes for the thymine found in DNA. A copy of the single stranded RNA can be made using reverse transcriptase (RT). Following this PCR amplification can also occur, with the whole process known as RT PCR.

Comparison of Cultural Methods of Detection with PCR

Currently dilution and plating methodology is still the most widely used technique for the isolation and detection of bacteria. However, there are distinct advantages of PCR as a detection technology, and also some disadvantages. Table 24-1 illustrates these differences. The biggest single advantage of PCR is that the molecular technique allows for the detection of viable but non-culturable bacteria. However, because PCR only detects nucleic acid sequences, there is always the possibility that a PCR-positive result occurs when the organism is not viable. PCR and subsequent sequence analysis does allow for specific identification of the target organism.

Polymerase chain reaction (PCR) amplification of DNA and RNA (Saiki et al., 1985; Mullis and Faloona, 1987) has become a key protocol in many biological laboratories. This DNA polymerase catalyzed reaction allows repeated synthesis of specific DNA sequences. A typical cycle involves denaturing the double stranded DNA into single strands, annealing short oligonucleotide primers to the single strands and extending the primer sequences using a DNA polymerase to complete the synthesis of strands complimentary to the original single strands. This cycling is repeated to obtain an exponential increase in the copies of the original DNA strand.

PCR allows amplification of specific DNA *in vitro*. The principle of the methodology involves the repetitive enzymatic synthesis of DNA, using two oligonucleotide primers that hybridize to opposite strands of DNA that flank the target DNA of interest. During each cycle, the number of copies of template DNA is theoretically doubled (Figure 24-1). In practice, 25 cycles of amplification results in approximately a million-fold increase in the number of DNA copies. The primers are often unique 18–25 base long oligonucleotides, carefully chosen to flank and allow amplification of the target DNA of interest. There are 3 steps in a PCR amplification cycle: i) template denaturation; ii) primer annealing; and iii) primer extension. All 3 steps occur at different but defined temperatures and time intervals. These repeating cycles are performed in an automated, self-contained temperature cycler or thermal cycler (Figure 24-2). The temperature cycle allows for precise temperature control required for each step.

Template denaturation occurs at a temperature greater than the melting temperature of the DNA (e.g., 94°C). Denaturation separates template DNA into single strands allowing subsequent primer annealing.

Table 24-1 Comparison of PCR and cultural methodology

Issue	PCR Technology	Cultural Methodology
Reduced time of detection	Yes	No
Increased sensitivity	Yes	No
Affected by PCR inhibitory substances	Yes	No
Detects only viable organisms	No	Yes
Detects viable but nonculturable organisms	Yes	No
Allows specific identification	Yes	No

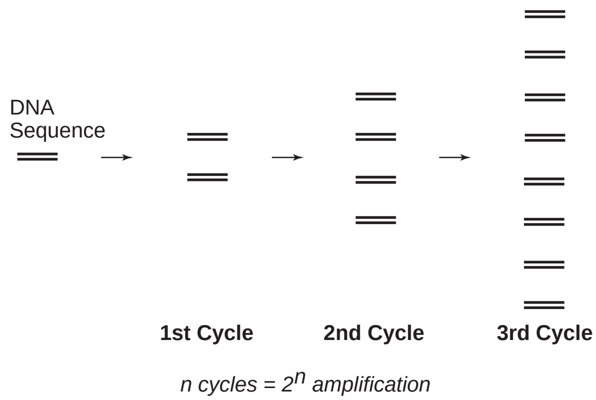


Figure 24-1 General schematic illustrating the theoretical amplification of DNA through the polymerase chain reaction. (From *Environmental Microbiology, A Laboratory Manual* © 1995, Academic Press, San Diego, CA.)



Figure 24-2 A thermal cycler that is used to conduct PCR using defined temperatures for specific time intervals. (Photo courtesy K.L. Josephson.)

Primer annealing occurs at a lower temperature which is typically 50–70°C. The higher the temperature of annealing, the more specific the annealing is, and the extent of annealing of mismatched primer to template is reduced. However, as primer annealing temperature increases, with a resulting beneficial increase in specificity, there is an associated decrease in sensitivity.

The final step of PCR is primer extension. Extension, which involves the synthesis of the DNA strand complementary to the template, extends from the 5′ and proceeds to the 3′ end of each primer and results in a double stranded copy of target DNA from each original single strand. Thus, a double stranded

DNA copy of the original target sequence results at the end of the first cycle. Primer extension typically occurs at 72°C and is catalyzed by the *Taq* DNA polymerase. *Taq* polymerase is a high temperature tolerant enzyme that was originally isolated from *Thermus aquaticus*.

The reaction components (template, primers, *Taq* polymerase, dNTPs, and the reaction buffer) are placed in a microfuge tube, and cycles of PCR amplification carried out in the thermal cycler.

Steps of the Polymerase Chain Reaction

There are three steps involved in amplification. These are denaturation, primer annealing, and extension.

Assume the double stranded segment to be amplified has the following sequence:

5′–AGC CGA TTA CGT ATG TTG AAT GTC GGC CCT–3′
3′–TCG GCT AAT GCA TAC AAC TTA CAG CCG GGA–5′

i) Denaturation

Denaturation involves separation of the two strands by heating (95–100°C), thereby breaking the hydrogen bonds which bind the two strands of DNA together. Consequently, the two strands will separate and we will have:

5′–AGC CGA TTA CGT ATG TTG AAT GTC GGC CCT–3′

3′–TCG GCT AAT GCA TAC AAC TTA CAG CCG GGA–5′

ii) Primer Annealing

If we then lower the temperature to 55°C, the strands will once again "hybridize" by forming hydrogen bonds—but if in the same solution we have a high concentration of 2 primers corresponding to the 3′ ends of each strand we will have the following:

```
5′–AGC CGA TTA CGT ATG TTG AAT GTC GGC CCT–3′
3′–                              A CAG CCG GGA–5′
                                 Primer A
  Primer B
5′–AGC CGA TTA C
3′–TCG GCT AAT GCA TAC AAC TTA CAG CCG GGA–5′
```

Note that the primers anneal on the 3′ ends of the template.

iii) Extension

The *Taq* polymerase can now recognize the primed strands, and attach to the double stranded portion. The optimum temperature for the *Taq* polymerase

to work is 72°C. Thus we have to increase the temperature from the previous 55°C to 72°C, and at this temperature the *Taq* polymerase will start constructing the complementary strands using the four deoxynucleotides (deoxyadenosine 5′-triphosphate (dATP), deoxycytosine 5′-triphosphate (dCTP), deoxyguanosine 5′-triphosphate (dGTP), and deoxythymidine 5′-triphosphate (dTTP)) which are also present in the solution We will then have:

5′–AGC CGA TTA CGT ATG TTG AAT GTC GGC CCT–3′
3′–TCG GCT AAT GCA TAC AAC TTA CAG CCG GGA–5′

5′–AGC CGA TTA CGT ATG TTG AAT GTC GGC CCT–3′
3′–TCG GCT AAT GCA TAC AAC TTA CAG CCG GGA–5′

The underlined sequences represent the extended or synthesized DNA, which occurs in the 3′ direction of the primers.

If we then repeat the whole process (the cycle) of heating to 95°C to separate the strands then lower the temperature to 55°C for the primers to anneal to the complementary strand and then raise the temperature again to 72°C for the *Taq* polymerase to work, we will see that after every cycle, we will have twice as many strands as we had in the previous cycle. The thermal cyclers can be programmed to automatically change the temperatures for a certain number of heating and cooling cycles (usually 25).

The amplified DNA is detected via gel electrophoresis and subsequent ethidium bromide staining. When viewed under a UV transilluminator, the DNA appears pink. Other strains such as Sybr Gold or Sybr Green II may also be used.

Advanced Topic: Sequence Analysis

In recent years identification of unknown bacterial isolates has been enhanced via PCR amplification of 16S rRNA gene sequencing and subsequent sequence analysis. Portions of the 16S rRNA gene sequences within bacteria are identical in all known bacteria. Conversely, between these homologous regions there are unique sequences of DNA associated with specific bacteria. So called "universal" primers have been designed that anneal to the conserved rRNA gene sequences, allowing amplification of the unique sequences within the conserved regions.

Aliquots of amplified gene that result from PCR with these universal primers can be sequenced inexpensively in commercial laboratories, or in many cases at university facilities. Once the sequence of the amplified rRNA is known, it can be used to identify the original bacterial source of RNA at the genus or species level. Computerized sequence databases have been compiled on large numbers of bacterial species. This allows for a comparison of an unknown rRNA gene sequence product with the known bacterial sequences that exist in the data base.

Several computer software sequence analysis programs are available to aid in sequence searches. One such data base is the Basic Local Alignment Search Tool (BLAST), which is provided by the National Center for

Biotechnology Information (NCBI) and is available on the Internet. This program allows researchers to perform sequence comparisons between known rRNA sequences and the amplified rRNA sequence of interest. For example, a sequence from an unknown soil isolate can be compared to all known sequences in the database, allowing for the isolate to be identified, or at least determine what organism it is most closely related to.

In this laboratory exercise, *E. coli* can be used as the target organism, or other known or unknown bacterial isolates can be analyzed.

Universal 16S rRNA Primers

8F	AGAGTTTGATCCTGGCTCAG	Universal bacterial 16S rRNA gene	Eden et al., 1991
1492R	TACGGY*TACCTTGTTACGACTT	Universal bacterial 16S rRNA gene	Wilson et al., 1990

*Y can be C or T, i.e., a degenerate base position.

24.3. PROCEDURE

First Period

Materials
1 ml 3–4 h *E. coli* broth culture (or other isolate)
PCR reaction tubes
microfuge tubes
microfuge
physiological saline (0.85% NaCl (w/v))
1 of each size micropipette with associated tips. (The following sizes will be needed: 0.5–10 µl, 2–20 µl, 20–200 µl, 100–1000 µl.)

1. Take 1 ml of a 3–4 hour broth culture of *E. coli* in a microfuge tube, and spin it down briefly using the microfuge. Wash the pellet in physiological saline, spin down, and resuspend in 1 ml physiological saline.

2. Make 3 serial, 10-fold dilutions of the *E. coli* cells by pipetting 0.1 ml of cells into 0.9-ml water blanks. Close caps on microfuge tubes. Assuming an original cell concentration of 10^8 cells ml^{-1}, we now have 10^8, 10^7, 10^6 and 10^5 cells ml^{-1} respectively in 4 microfuge tubes.

3. Make a master mix for 6 PCR reactions (to ensure sufficient reagent for 5 reactions since a small amount is lost in the pipette tips) in a microfuge tube as summarized in Table 24-2. The master mix contains deoxynucleotides, 2 primers, buffer, and water necessary for 6 reactions. The 5 reactions will be the 4 concentrations of cells from above in step 2 and a negative control.

4. Aliquot 35 µl of master mix into each of 5 PCR reaction tubes. Close the tubes and label 1 to 5. Note: Some older thermal cyclers require addition of an overlay of mineral oil to prevent evaporation.

Table 24-2 Master mix composition for the PCR procedure

Component	Kit Concentration	Volume per Tube (µl)	Volume for 6 tubes (µl)	Final Concentration
Buffer	10X	5	30	1×
dATP	10 m*M*	1	6	200 µM
dCTP	10 m*M*	1	6	200 µM
dGTP	10 m*M*	1	6	200 µM
dTTP	10 m*M*	1	6	200 µM
Primer 1[a]	10 µM	2.5	15	0.5 µM
Primer 2[a]	10 µM	2.5	15	0.5 µM
Sterile H_2O		21.0	126	
Total Volume[b]		35	210	

[a]Primers need to be synthesized ahead of time. Most large universities have a biotechnology center with synthesizing capabilities. Commercial companies also synthesize primers.
[b]Assumes a final volume of 50 µl after addition of 10 µl of template and 5 µl of enzyme.

5. Add 10 µl of *E. coli* dilutions to tubes 1 through 4. Use new pipette tips for each tube. To the 5th tube (the negative control), add 10 µl of sterile water.

6. In a microfuge tube, prepare enough diluted enzyme solution for 6 tubes by taking 3 µl of stock *Taq* at 5 U µl^{-1} and adding 27 µl sterile water.

7. Lyse the cells in the tubes of step 5 by heating in the thermal cycler at 98°C for 10 min. After cooling to 25°C, add 5 µl of diluted *Taq* enzyme to each tube. (See Table 24-3 for a check list of all components per tube.)

8. In these tubes, we therefore have 10^6, 10^5, 10^4, and 10^3 cells, respectively [10 µl × (original # cells present/1000 µl)]. The 5th tube is the negative control which has no DNA template.

9. Place the tubes in the thermal cycle which has previously been programmed (Figure 24-2). The template DNA is initially denatured at 94°C for 1.5 min, followed by a 1 min annealing at 37°C and extension for 1 min at 72°C. This is followed by 25 cycles of PCR at 94°C for 1.5 min with 1 min annealing at 55°C and 1 min extension at 72°C. For the last cycle, the extension step requires 7 min to complete synthesis of all strands. The entire process should take approximately 2 h. The thermal cycler will hold the samples at 4°C until removed.

Table 24-3 Checklist for all component tubes

Component	Volume (µl)
dNTPs	4 (1 of each)
Buffer	5
H_2O	21
Primers	5 (2.5 of each)
Enzyme	5
Template	10
	50 µl total

Second Period

Materials
PCR product from Period 1
0.64 g agarose
laboratory balance (±0.001 g)
125-mL Erlenmeyer flask

40 ml 1× TBE buffer
microwave oven
electrophoresis gel casting tray with comb
microfuge tubes
1 of each size micropipette with associated tips (The following sizes will be needed: 0.5–10 µl, 2–20 µl, 20–200 µl, 100–1000 µl)
Ficoll loading buffer
123 bp DNA ladder stock
power source for electrophoresis
electrophoresis gel apparatus with electrodes (Figure 24-3)
1 µg ml^{-1} ethidium bromide
protective gloves
ethidium bromide liquid and solid disposal containers
UV transilluminator (Wavelength = 302 nm)
UV-protective goggles
darkroom for UV observation
instant film camera and film (Alternatively, a gel imaging system and thermal printer may be used)

1. Prepare a 1.6% agarose gel by weighing out 0.64 g of agarose in a 125-ml Erlenmeyer flask and adding 40 ml of 1× TBE buffer. Place in microwave oven for 1 min at high power. Remove from microwave and swirl to make sure all the agarose has melted, otherwise reheat for another minute.

2. Cool melted agarose to approximately 50°C. Pour molten gel into the electrothesis gel casting tray making sure no bubbles are trapped within the agarose. Place comb into gel and wait for approximately 20–30 min for the gel to solidify (Figure 24-3).

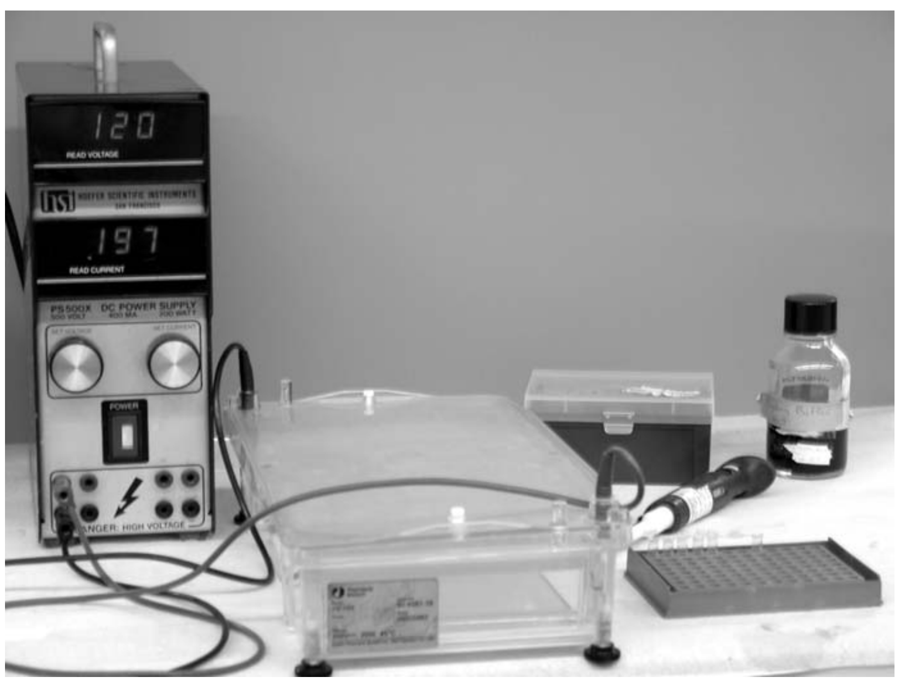


Figure 24-3 Micropipettes and electrophoresis equipment needed for PCR amplifications and subsequent DNA detection. (Photo courtesy K.L. Josephson.)

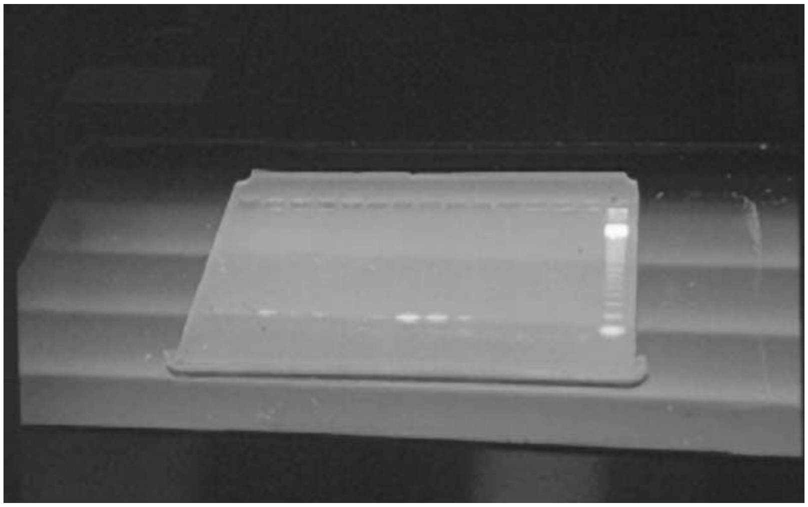


Figure 24-4 A gel illustrating stained DNA that can be seen under UV light. On the right is a 123 bp size marker. (Photo courtesy © Robert Walker.)

3. Remove comb carefully, place gel in electrophoresis chamber. Add 1× TBE buffer until the gel is completely submerged. The comb creates wells in the gel to accommodate samples.

4. Label a fresh set of microfuge tubes 1 through 5 and place 5 µl of loading buffer in each. Add 15 µl of PCR sample to tubes 1 through 5. To a 6th tube add 20 µl of a 123 bp DNA ladder which already contains loading buffer. Carefully load 20 µl of each sample into wells within the gel.

5. Connect the electrodes to the power source and run the gel under constant current at 100 V for 1.5–2 h.

6. Turn off the power source, disconnect electrodes and carefully transport gel into the staining tray.

7. Cover gel with ethidium bromide (EtBr) solution (1 $\mu g\, ml^{-1}$) and allow to stain for 15 min. Recycle the EtBr by decanting the EtBr back into the container. Rinse the gel 2–3 times with tap water. Place ethidium bromide washings in the waste container.

8. De-stain excess EtBr by soaking in water for 20–30 minutes.

9. Place gel on the transilluminator and observe DNA bands in the presence of UV light. Photograph the gel. Compare the size of the amplified product to the DNA size marker (Figure 24-4).

10. In addition, place microfuge tubes with amplified DNA on ice and ship or transport to a commercial laboratory or university facility for sequence analysis.

Third Period

1. Sufficient time should be allowed between Periods 2 and 3 for information on the sequence analysis to be obtained.

2. It is beyond the scope of this lab manual to give detailed information on sequence analysis, but programs such as BLAST (Altshul et al., 1990) can be found on the National Center for Biotechnology Information's World Wide Website (http://www.ncbi.nem.nih.gov). We strongly advise that the instructor for this class have experience in sequence analysis if this analysis is to be attempted. Note that computers need to be provided to students, or the analysis can be done outside of the normal lab hours.

3. Comparison of the target amplified DNA sequence with known DNA sequences allows for the identification of the target organism at the genus level.

24.4. TRICKS OF THE TRADE

DO:

- Wear disposable rubber gloves to avoid sample contamination of DNA
- Remember to add all reagents to master mix
- Use a new pipette tip for each dilution step
- Close all microfuge caps tightly
- Make sure the positive (red) electrode is at the opposite end to the loading wells during electrophoresis, or the DNA will travel in the wrong direction

DO NOT:

- Allow contents of flask to boil over while preparing agarose
- Remove comb from electrophoresis chamber until agarose is completely set
- Allow electrophoresis to occur for excessive periods of time so that the amplified DNA exits the gel and is lost

24.5. POTENTIAL HAZARDS

DO:

- Be careful handling hot agarose
- Wear gloves and avoid skin contact with ethidium bromide since this is a mutagen. For accidental skin contact wash area with copious amounts of water
- Wear protective goggles when observing the gel since UV light is dangerous to the eyes

24.6. QUESTIONS AND PROBLEMS

1. Assuming your original stock *E. coli* suspension had 10^8 CFU ml^{-1}, what was the minimum number of CFU that you could detect, i.e., what was sensitivity in terms of CFUs?

2. What was the size of your amplification product based on a comparison with the DNA ladder?

3. How did you reduce sample contamination throughout the experiment?

4. Was your negative control negative? If not, why not?

5. Was the sequence analysis successful?

24.7. REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Eden, P.A., Schmidt, T.M., Blakemore, R.P., and Pace, N.R. (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *International Journal of Systematic Bacteriology* **41**, 324–325.

Mullis, K.B., and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods in Enzymology* **225**, 335–350.

Saiki, R.K., Scarf, S., Faloona, F.A., Mullis, K.B., Hoen, G.T., Erlich, H.A., and Arnheim, N. (1985) Enzymatic amplification of beta-globulin genomic sequences and restriction size analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350–1354.

Wilson, D.H., Blitchington, R.B., and Green, R.C. (1990) Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of Clinical Microbiology* **28**, 1942–1946.

APPENDIX 1

Preparation of Media and Stains for Each Experiment



Preparation of Media and Stains for Each Experiment

APPENDIX 1

EXPERIMENT 1

Physiological Saline

0.85% (w/v) NaCl.

Trypticase Soy Broth

May be obtained from various manufacturers. Follow the manufacturer's directions for preparation of the broth.

EXPERIMENT 2

None utilized.

EXPERIMENT 3

Phenolic Rose Bengal Stain

Prepare under a fume hood as phenol vapors are hazardous. To 100 ml of a 5% aqueous solution of phenol, add 1.0 g Rose Bengal and 0.03 g $CaCl_2$.

EXPERIMENT 4

Lactophenol Mounting Fluid

10 g phenol crystals; 20 g lactic acid; 40 g glycerol; 20 ml deionized water. Prepare only under a fume hood as phenol vapors are hazardous. Dissolve the above with gentle heat, then add 0.05 g cotton blue.

Rose Bengal-Streptomycin Agar

10 g glucose; 5 g peptone (a meat or dairy by-product); 1 g K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.033 g Rose Bengal; 15 g agar; 1000 ml tap water. After autoclaving the agar at 21 psi (140 kPa) for 15 min and cooling to ca. 45°C, add streptomycin in the form of a filter-sterilized solution to make the final concentration of the antibiotic 30 $\mu g\,mL^{-1}$. Streptomycin is an antibiotic which inhibits the initiation of protein synthesis and causes misreading of mRNA in prokaryotes (Stryer, 1988). Rose Bengal is a phenolic compound which not only has staining properties, but also inhibits bacterial growth and excessive spreading of certain types of fungi. Streptomycin is heat labile.

EXPERIMENT 5

Glycerol-Casein Agar

In 1000 ml of deionized water dissolve the following: 10.0 g glycerol (corresponds to 8.5 ml), 0.3 g vitamin-free casein, 2.0 g KNO_3, 2.0 g NaCl, 2.0 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 18 g agar, and 50 mg cycloheximide (heat stable). Adjust to pH 7.0 with concentrated HCl after autoclaving at 21 psi (140 kPa) for 15 min (wear protective eyewear and gloves and do this in a fume hood!).

Gram Stain Reagents

i) Crystal Violet

Gram primary stain. 0.4%. Crystal violet in an aqueous alcohol solution. Harmful or fatal if swallowed. Combustible: Avoid open flames and use in a well ventilated area. May cause eye irritation—avoid eye contact.

ii) Decolorizer

1 : 3 (v/v) acetone : isopropyl alcohol. Causes gastrointestinal difficulties if swallowed. Combustible: Keep away from flame sources.

iii) Iodine Mordant

Harmful or fatal if swallowed. May cause eye irrigation—avoid eye contact. 13% polyvinylpyrrolidone-iodine complex in 1.9% aqueous KI.

iv) Safranin

Gram counterstain. 0.25% safranin in 20% ethyl alcohol. Harmful or fatal if swallowed. Combustible—avoid open flames and use in a well ventilated area.

Nutrient Broth

May be obtained from various microbiological supply houses. Follow the manufacturer's directions for preparation of the broth.

Peptone-Yeast Agar

In 1000 ml deionized water add: 5 g peptone, 3 g yeast extract, and 15 g agar. After autoclaving at 21 psi (140 kPa) for 15 min and after the agar has cooled to ca. 45°C, add 10 ml of 1.0 M $CaCl_2$ to make the solution 10 m*M* in $CaCl_2$ (adding the $CaCl_2$ to hot agar causes flocculation). Adjust the pH to 7.0 with concentrated HCl (wear protective eyewear and gloves and do this in a fume hood!) after autoclaving.

R_2A Media

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation of the agar.

EXPERIMENT 6

Bristol's Solution

In 1000 ml tap water dissolve: 0.25 g $NaNO_3$, 0.025 g $CaCl_2$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.075 g K_2HPO_4, 0.018 g KH_2PO_4, 0.025 g NaCl, and 0.5 mg FeCl.

EXPERIMENT 7

None utilized.

EXPERIMENT 8

2,3,5-Triphenyltetrazolium chloride (TTC), 3%

Dissolve 3 g TTC in about 60 ml deionized water in a 100 ml volumetric flask. Add water to mark. (Not all of the TTC will dissolve, so the solution should be filtered through filter paper.) This compound is photosensitive to short wave UV, which will lead to reduction to TPF—avoid exposure to excessive light.

Triphenyl formazan (TPF)

Standard Stock Solution 100 mg L^{-1}: Dissolve 100 mg TPF in about 500 ml methanol in a 1 L volumetric flask. Dilute to mark with methanol. TPF will isomerize to a yellow form if exposed to excessive light (*cis-trans* isomerization), but will revert to the red form if returned to the dark.

EXPERIMENT 9

None utilized.

EXPERIMENT 10

2,4-D Indicator Plates

The 2,4-D indicator plates contain (per liter of distilled water) 112 mg of $MgSO_4 \cdot 7H_2O$, 5 mg of $ZnSO_4 \cdot 7H_2O$, 2.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 218 mg of K_2HPO_4, 14 mg of $CaCl_2 \cdot 2H_2O$, 0.22 mg of $FeCl_3 \cdot 6H_2O$, 0.5 g of NH_4Cl, 500 mg of 2,4-D, 80 mg of eosin B, 13 mg of methylene blue, and 20 g of purified agar.

2,4-D Screening Broth

500 ml 2 MSB for 2,4-D Screening Broth (see below), 100 ml of 0.04% (w/v) solution of bromthymol blue dye, 350 ml deionized H_2O. Adjust the pH to 7.0 with 1 *M* HCl before autoclaving at 21 psi (140 kPa) for 20 min. Make the solution 500 mg L^{-1} 2,4-D.

2 MSB (Minimal Salts Broth) for 2,4-D Screening Broth (2 concentration)

224 mg $MgSO_4 \cdot 7H_2O$, 10 mg (10 ml of 0.1% (w/v) stock solution) $ZnSO_4 \cdot 7H_2O$, 5 mg (ml of 0.1% (w/v) stock solution) $Na_2MoO_4 \cdot 2H_2O$, 680 mg KH_2PO_4, 710 mg Na_2HPO_4, 28 mg $CaCl_2 \cdot 2H_2O$, 0.44 mg (440 µl of 0.1% (w/v) stock solution) $FeCl_3 \cdot 6H_2O$, 1.00 g NH_4Cl. Add ingredients individually to deionized H_2O allowing each compound to dissolve completely before adding the next. Adjust pH to 7.0 with 1 *M* HCl. Bring the final volume to 1000 ml. Autoclave at 21 psi (140 kPa) for 20 min. The solution may precipitate out slightly after autoclaving; simply shake the solution well before using for media preparation.

Endo Agar

May be obtained pre-prepared from various microbiological supply houses. Follow the manufacturer's directions for preparation of agar.

Eosin-Methylene Blue (EMB)/2,4-D Agar

500 mg L^{-1} 2 MSB for EMB. Add the following from available stock solutions to achieve the denoted concentrations of each in the final volume (1000 ml): 50 mg L^{-1} yeast extract, 80 mg L^{-1} Eosin B, 13 mg L^{-1} Methylene Blue. Adjust the pH to 7.0 by adding 1 *M* HCl. Then, add 20 g purified (Noble) agar. Add deionized H_2O to bring the final volume to 1000 ml. Heat to dissolve the agar under constant stirring and auto-clave at 21 psi (140 kPa) for 20 min. Add 50 ml 1% (w/v), filter-sterilized, 2,4-D stock solution to make the final agar 500 mg L^{-1} in 2,4-D. ***Do not autoclave, 2,4-D-containing wastes as the herbicide may volatilize into the air!***

Lactose Broth

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation. Double Strength Lactose Broth (DSLB) is made at twice the concentration of Single Strength Lactose Broth (SSLB).

Levine's Eosine-Methylene Blue (EMB) Agar

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation.

Minimal Salts Broth for EMB (2× Concentration) (2×MSB for EMB)

224 mg $MgSO_4 \cdot 7H_2O$, 10 mg (10 ml of 0.1% (w/v) stock solution) $ZnSO_4 \cdot 7H_2O$, 5 mg (5 ml of 0.1% (w/v) stock solution) $Na_2MoO_4 \cdot 2H_2O$, 435 mg KH_2PO_4, 28 mg $CaCl_2 \cdot 2H_2O$, 0.44 mg (440 µl of 0.1% (w/v) stock solution) $FeCl_3 \cdot 6H_2O$, 1.00 g NH_4Cl. Add the ingredients individually to deionized H_2O allowing each compound to dissolve completely before adding the nest. Adjust the pH to 7.0 with 1 *M* HCl. Bring the final volume to 1000 ml. The solution may precipitate out slightly after autoclaving; simply shake the solution well before using for media preparation.

Peptone-Yeast Agar

In 1000 ml deionized water add: 5 g peptone, 3 g yeast extract, and 15 g agar. Adjust the pH to 7.0 with concentrated HCl (wear protective eyewear and gloves and do this in a fume hood!) After autoclaving at 21 psi (140 kPa) for 15 min and after the agar has cooled to ca. 45°C, add 10 ml of 1.0 M $CaCl_2$ to make the solution 10 m*M* in $CaCl_2$ (adding the $CaCl_2$ to hot agar causes flocculation).

Peptone Yeast Extract/Hg (PY/Hg) Agar

In 1000 ml deionized water add: 5.0 g peptone, 3.0 g yeast extract, and 15 g agar. Adjust the pH to 7.0 with 1 *M* HCl. After autoclaving at 21 psi (140 kPa) for 20 min and after the agar has cooled to ca. 45°C, add 10 ml of sterile, autoclaved 1.0 *M* $CaCl_2$ to make the solution 10 m*M* in $CaCl_2$ (adding the $CaCl_2$ to hot agar causes flocculation). Filter sterilize 5 ml of 6.75 g L^{-1} $HgCl_2$ stock solution and add to the cooler agar. ***Do not autoclave Hg-containing substances as Hg vapors will be released into the air!***

EXPERIMENT 11

Peptone Yeast Agar

In 1000 ml deionized water add: 5 g peptone, 3 g yeast extract, and 15 g agar. Adjust the pH to 7.0 with concentrated HCl (wear protective eyewear and gloves and do this in a fume hood!) After autoclaving at 21 psi (140 kPa) for 15 min and after the agar has cooled to ca. 45°C, add 10 ml of 1.0 M $CaCl_2$ to make the solution 10 m*M* $CaCl_2$ (adding the $CaCl_2$ to hot agar causes flocculation).

Peptone Yeast Agar with Cd

Prepare normal peptone yeast agar. After autoclaving, amend with 500 µg Cd ml^{-1} using Cd $(NO_3)_2 \cdot 4H_2O$.

EXPERIMENT 12

Dissolved Oxygen Test Kit

Hach Company, Loveland, Colorado

Phenol Solution

10 g L^{-1} 2,4-dichlorophenol. If necessary, 2,6-dichlorophenol may be substituted.

EXPERIMENT 13

R_2A Media

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation of the agar.

EXPERIMENT 14

Hach Buffer Nutrient Pillows

Poly Seed Microorganism Inoculum Capsule, 2-chloro-6-(trichloromethyl) pyrimidine (TCMP). Chlorine Test Kit, free and total, model CN-80. DPD Total Chlorine Reagent Pillows.

Hach Company, Loveland, Colorado

EXPERIMENT 15

mEndo Broth-MF

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation of the broth.

mFC Agar

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation. Other reagents may need to be purchased to prepare the medium. For example, the product sold by Difco Laboratories (Detroit, Michigan) requires the addition of rosolic acid.

EXPERIMENT 16

Lactose Broth

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation. Double Strength Lactose Broth (DSLB) is made at twice the concentration of Single Strength Lactose Broth (SSLB).

Levine's Eosine-Methylene Blue (EMB) Agar

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation.

Endo Agar

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation.

EXPERIMENT 17

Nutrient Agar

May be obtained pre-prepared from various microbiological supply houses. Follow the manufacturer's directions for preparation of the agar.

Tris-Buffered Saline

1× TBS–1 (10× Tris): 1600 ml double distilled water, 63.2 g Trizma® base (sigma Chemical Company, St. Louis, Missouri), 163.6 g NaCl, 7.46 KCl, 1.13 g Na_2HPO_4. Adjust to pH 7.2–7.4 with 6 MHCl. Tris (1×): 3680 ml of double distilled water, 320 ml of TBS–1. Check to verify that the final pH is in the range 7.2–7.4. If necessary, adjust with HCl or NaOH.

Trypticase Soy Agar

Add 0.1 g of Peptone to 100 mls of distilled water. Mix to dissolve and autoclave. Alternatively, Nutrient Broth can be used (see Experiment 5).

EXPERIMENT 18

Peptone Water

Add 0.1 g of Peptone to 100 mls of distilled water. Mix to dissolve and autoclave. Alternatively, Nutrient Broth can be used (see Experiment 5).

EXPERIMENT 19

Colilert Powdered Media

May be obtained from Environetics, Inc., Branford, Connecticut.

EXPERIMENT 20

Demonstration only.

EXPERIMENT 21

Elution Buffer

1 g Laureth-12 in a glass beaker and add 100 ml of distilled water. Heat the beaker to melt the Laureth-12 and transfer the solution to a 1000 ml volumetric flask. Rinse the beaker several times to ensure that all the detergent was transferred to the flask. Add 10 ml of Tris buffered saline solution, pH 7.4, 2 ml of EDTA solution, pH 8.0; and 150 µl of antifoam A. Bring solution to a final volume of 1000 ml with distilled water. The buffer will have an opaque appearance.

Eluting Solution

300 ml 1% (w/v) SDS, 300 ml 1% (v/v) Tween® 80, 240 ml 10× PBS, (see below), 2160 ml H_2O, 0.3 ml Antifoam A (Sigma Chemical Company, St. Louis, Missouri). Mix well and adjust the pH to 7.4 with 1 *M* HCl. Makes 3 L. Use this quantity to elute filters.

10× Phosphate Buffered Saline (PBS)

80 g NaCl, 2 g KH_2PO_4, 12.72 g Na_2HPO_4, 2 g KCl. Bring the final volume to 1 L. **1× PBS**—Dilute 10× PBS with 9 volumes water and adjust the pH to 7.4 with 0.1 *M* HCl or 0.1 *M* NaOH.

SDS (Sodium dodecyl sulfate)

May be obtained from various manufacturers.

EXPERIMENT 22

Nutrient or Typticase Soy Agar

May be obtained from various manufacturers. Follow manufacturer's directions for preparation.

Phosphate Buffered Saline

80 g NaCl, 2 g KH_2PO_4, 12.72 g Na_2HPO_4, 2 g KCl. Bring the final volume to 1 L. **1× PBS**—Dilute 10× PBS with 9 volumes water and adjust the pH to 7.4 with 0.1 *M* HCl or 0.1 *M* NaOH.

EXPERIMENT 23

Anti-Foam B

Sigma Chemical Company, St. Louis, Missouri.

Nutrient Agar (NA) or Trypticase Soy Agar (TSA)

May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation.

Sabouraud Dextrose Agar (SDA)

Difco, Detroit, MI. May be obtained as a mix from various microbiological supply houses. Follow the manufacturer's directions for preparation.

EXPERIMENT 24

123 bp DNA Ladder Stock

20 µl 123 bp DNA ladder (GIBCO BRL, Gaithersburg, Maryland, 0.8 µg L^{-1} original concentration), 25 µl Ficoll loading buffer, 50 µl deionized water. Use 10 µl in each gel lane.

Ethidium Bromide (EtBr)

A hazardous mutagen. Ethidium bromide stains DNA by intercalating within the double stranded helix. As there is no difference in the basic chemical properties of the bacterial DNA being stained in the reaction tubes and the DNA found in the cells of the students performing the experiment, extreme caution must be exercised to avoid all bodily contact with ethidium bromide! 1 µg ml^{-1}. Add 40 µl of 10 mg ml^{-1} stock solution to 400 ml water.

Ficoll Loading Buffer

20% Ficoll type 400 (approximate MW = 400,000), 0.1 *M* Na_2EDTA, pH 8.0, 1% (w/v) SDS, 0.25% (w/v) bromphenol blue. Dissolve the components on low heat for 1 h to dissolve the Ficoll.

Physiological Saline

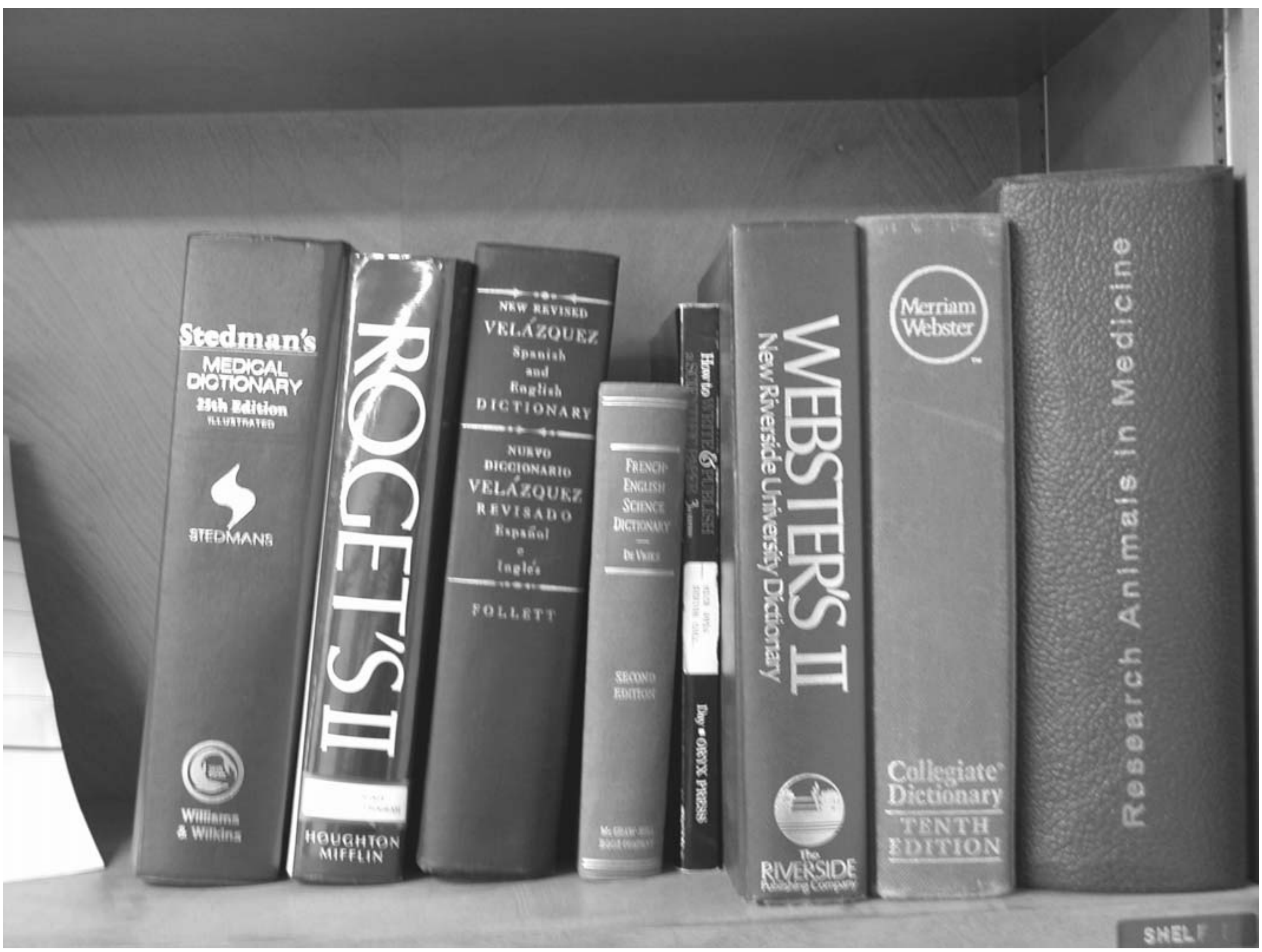
0.85% (w/v) NaCl.

Tris-Borate-TBE Buffer (1×)

0.09 *M* Tris, 0.09 *M* Na_3BO_3, 0.001 *M* Na_4EDTA.

APPENDIX 2

Glossary



Glossary

APPENDIX 2

absorbance The transfer of energy from light to electrons in molecules and atoms.

acid According to the Brønsted definition, an acid is any substance which donates a proton (H^+) in solution.

adsorbed *With respect to water*: Water that is bound by electrostatic attraction to the charged surface on a soil colloid is said to be adsorbed to the surface or to the colloid (not taken into the colloid).

aerobic The use of oxygen as a terminal electron acceptor.

agar A polysaccharide composed of pentose sugars extracted from kelp and widely used as a substrate for containing nutrients for culturing microorganisms in dilution and plating experiments.

agarose A highly purified form of agar.

anabolic reaction A reaction that results in the formation of more complex, reduced substances, such as forming starch from CO_2.

anaerobic Combined oxygen such as nitrate or sulphate is used as a terminal electron acceptor, rather than oxygen.

anion An ion that is negatively charged.

antibiotic disk A fibrous disk saturated with a solution of antibiotic.

aseptic hood A type of hood which often uses filters and air flow to exclude microorganisms from the work area.

assimilatory nitrate reduction Reduction of nitrate by organisms to ammonium, which is used by the organism in the formation of nitrogen-containing compounds such as protein.

autoclave A device which uses steam under pressure to sterilize materials at elevated temperatures.

autotrophic An organism that can synthesize all of its needed energy from inorganic sources, e.g., plants, algae.

bacterial lawn An even layer of bacterial growth across the surface of an agar plate often used to diagnose or enumerate viruses in a sample by observing for plaques.

bacteriophage A virus which replicates by using bacteria as a host.

base *In nucleic acids*: One of the nitrogenous bases found in nucleic acids: adenine, thymine, cytosine, guanine, or uracil.

base pair A unit of two nucleotides, each from opposite DNA molecules in a strand of double stranded DNA.

bioassay An assay performed by analyzing the effects of an agent or treatment on living organisms.

biodegradable A substance that can be metabolized by or broken down by microorganisms.

biological oxygen demand (BOD) The amount of oxygen in an environmental sample such as water, that is needed to oxidize organic residues within the sample.

Bristol's solution A solution containing only mineral substances and no oxidizable organic material for culturing or enumerating algae.

broth media Microbial media in a solution form as opposed to a solid medium, such as agar.

bulk density A measurement of the density of soil as it is found in the environment, on a dry mass basis.

capsid Protein coat surrounding a virus.

capsomer Protein subunits that make up the capsid of a virus.

catabolic reaction A reaction involving the oxidation of more complex and reduced substances to simpler, more oxidized substances, usually with an accompanying release of energy utilized by a living organism.

cation exchange capacity The ability of soil particles (negatively charged) to bind cations electrostatically and retain them on their surfaces. Usually expressed as mmol (+) (positive charge) kg^{-1} dry soil.

cation A positively charged ion.

cell monolayer Animal cells one cell-layer deep, grown in a culture flask and used to analyze samples for viruses by enabling observation for cytopathogenic effect (CPE).

chemoautotrophic An autotrophic organism that derives its energy by converting the energy derived from oxidizing reduced inorganic compounds to ozidized forms. For example, *Nitrosomonas* and *Nitrobacter* are chemoautotrophic organisms involved in the nitrogen cycle.

chloroplast An organelle of photosynthetic organisms such as algae and higher plants which captures light energy and converts it into chemical energy in the form of carbohydrates.

clay The smallest of the three textural size fractions of soil: soil particles that are smaller than 0.002 mm in diameter (USDA system).

coccus Spherical-shaped bacterium.

coefficient of variation A measure of the variability of data in a data set. It is equal to the sample standard deviation divided by the sample mean and is expressed as a percentage.

coenzyme A substance that participates in chemical reactions that must first react with a substrate before the substrate can bind to an enzyme.

cofactor An accessary to enzymes which binds to an enzyme, assisting in activating or inactivating its binding site. Cofactors may be as complex as organic compounds or as simple as metal ions.

coliform All aerobic and facultatively anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria which ferment lactose with gas production in prescribed media within 48 hours at 35°C. This group includes *Escherichia coli, Enterobacter*, and *Klebsiella*.

Colilert® Commercial defined substrate technology (DST) test approved by the U.S. Environmental Protection Agency for drinking water analysis. Colilert® is a registered trademark of Environetics, Inc., Branford, Connecticut.

coliphage A virus which uses coliform bacteria as a host for replication.

colloid In soils: an inorganic or organic particle that is <0.002 mm in diameter.

colony forming unit (CFU) The microbiological entity that reproduces to form a colony in culture. The CFU may have been a single cell, a group of cells aggregated together, a spore, or a segment of fungal hypha.

colony Generally, a macroscopic mass of a single type of microorganism in culture, although some colonies may be fixed organisms.

confirmed test The second stage of the coliform MPN where tubes screening positive in the presumptive test are further examined to confirm that the organisms present in the water sample were indeed coliforms.

conjugation The exchange of genetic material via cell/cell contact.

contact slide A qualitative assay involving the burying of a slide in soil, incubation, removal of the slide, and microscopic analysis of the slide for the examination of types of microorganisms adhering to the slide and their spacial interrelationships.

counterstain A second stain applied after an initial stain has possibly been removed. In the Gram stain process, safranin serves as the counterstain, adhering to those organisms where the crystal violet stain does not.

cryptosporidiosis An infection by the organism *Cryptosporidium*.

cuvette A transparent container holding a sample for analysis is spectrophotometry. Cuvettes for visible absorption spectrophotometry may be

made of glass while cuvettes for UV absorption spectrophotometry must be made of quartz due to absorption of UV by glass.

cyst A dormant stage of protozoa which tends to be resistant to harsh environments and disinfection and still capable of infection.

cytopathogenic effect (CPE) Cell destruction in virus cultures due to infection by a virus. Observed CPA serves as positive confirmation of the presence of a virus specific to the type of cells and conditions used.

death phase of growth Net loss of cells in culture.

decolorize To remove stain. In the Gram stain process, the sample organisms are decolorized to remove the crystal violet stain prior to counterstaining with safranin.

defined substrate technology (DST) A new approach for the simultaneous detection, specific identification, and confirmation of total coliforms and *Escherichia coli* in water.

dehydrogenase An enzyme which catalyzes both hydrogenation and dehydrogenation reactions, i.e., those reactions involving reducing a substrate by adding H^+ and 2 electrons (hydrogenation) or oxidizing a substance by removing H^+ and 2 electrons (dehydrogenation).

denaturation *In PCR*: Inducing double stranded DNA to separate into two single strands of DNA through the addition of heat.

denitrification The process of microbial reduction of nitrate to reduced forms of nitrogen, such as dinitrogen gas or N_2O.

dilution series A series of subsequent dilutions used to make solutions much more dilute in a substance or microorganism.

dinitrogen gas Elemental nitrogen, N_2.

direct count A method for enumerating organisms (usually bacteria) in soil by filtering a known aliquot of suspension and counting the stained organisms under a microscope.

dissimilatory nitrate reduction Reduction of nitrate where the reduced products are discarded by the organism as waste, often in the form of N_2 and N_2O. See *assimilatory nitrate reduction*.

DNA ladder A mixture of standard DNA polymers that vary by a fixed increment of base pairs, e.g., 123 bp.

DNA polymerase An enzyme which catalyzes the polymerization of the second strand of DNA from the template strand.

dNTP A generic abbreviation for the triphosphate of any deoxynucleoside.

DSLB **D**ouble **S**trength **L**actose **B**roth. A medium used in the presumptive test of the coliform MPHN test. See also *SSLB*.

Durham tube A small, inverted, liquid-filled test tube for collecting gas formed by microbial metabolism in broth cultures. The Durham test is used to test for the presence of organisms capable of metabolizing the broth nutrients under the given conditions through observation for gas present in the tube.

electrophoresis A method of separating particles, usually large macromolecules, based on their mass charge ratio, by placing the particles in an electric field under a set voltage and separating through a "molecular sieve" of a polymer network as is found in an agarose gel.

electrostatic attraction When positively charged particles attract or are attracted by negatively charged particles.

elemental sulfur Elemental sulfur, S^0.

endo agar A medium used in the confirmed test of the coliform MPN.

enrichment culture A procedure for selecting for a particular type of microorganism based on its ability to utilize one particular source of carbon, such as an herbicide.

enteric Of or pertaining to the intestines.

enteric viruses Viruses which multiply in the intestinal tract.

enzyme A biological catalyst made of protein.

ethidium bromide An intercalating dye used to detect nucleic acid when viewed under UV light.

eukaryotic Organisms characterized as having genetic material condensed into a nucleus, and containing cell organelles.

excystation Production of a vegetative form of protozoon from a cyst.

exponential phase of growth A finite period of time where microbial growth in culture is logarithmic due to binary fission.

extension *In PCR*: The process of adding nucleotides to the primer in reactions catalyzed by a DNA polymerase. Extension occurs 5′ to 3′ on the daughter strand.

extracellular enzyme An enzyme which is excreted by a cell to perform specific functions outside of the cell.

facultative anaerobic An organism that can use oxygen as a terminal electron acceptor if it is available, or otherwise use the other terminal electron acceptors, such as nitrate.

fecal coliform Bacteria with ability to ferment lactose with the production of acid and gas at 44.5°C within 24 hours.

fermentation Anaerobic metabolism.

field capacity The water content of a wetted soil that has been allowed to drain for two days, associated with a surface tension of –1/3 bar.

flame To sterilize a microbiological implement, often by immersing it in ethanol and igniting in the flame of a gas or alcohol burner, or even simply exposing the object to the heat of a flame.

flocculation In water purification: The process of removing particulate matter from water by treating it with a flocculent, such as alum (aluminum sulfate), forming a precipitate (floc) which entraps the particulate material, making it easy to remove fine particles from the water using a course filter, such as a sand filter.

fluorescence The re-radiation of light at a lower energy level (longer wavelength) than what was absorbed. For example, some compounds, such as ethidium bromide, absorb invisible, high-energy UV-light and re-radiate it as lower energy visible light.

glycerol-casein agar A medium containing nutrient sources difficult for most bacteria to metabolize and are hence used for the isolation/selection/enumeration of actinomycetes.

Gram-negative organisms: Organisms that appear red under the microscope after Gram staining.

Gram-positive organisms: Organisms that appear blue under the microscope after Gram staining.

Gram stain A diagnostic staining procedure for bacteria which separates bacteria into two classes: gram-positive and gram-negative. The procedure is based on the difference in cell wall composition and structure among bacteria. In the basic procedure, bacteria are fixed to a slide and treated with a blue dye, such as crystal violet. After treatment with the mordant iodine, the stained cells are processed with a decolorizing agent and counter stained with a red stain, safranin. Gram-positive bacteria have a single-layered cell wall which is dyed by the crystal violet and cannot be decolorized. Therefore, these organisms are blue after Gram staining. Gram-negative bacteria, on the other hand, have a multilayered cell wall that is not stained well by the crystal violet which is easily removed. Thus, after decolorizing and counterstaining with safranin, these organisms appear red.

greenhouse gas A gas, such as CO_2 or N_2O, which absorbs wavelengths of sunlight which would otherwise be re-radiated into space, thereby believed to be an agent in increasing the average temperature of the atmosphere.

heavy metal A metal, often with high atomic mass, that often irreversibly interferes with metabolism, poisoning the ingesting organism. Examples include Cr, Hg, Ni, and Pb.

heterotrophic An organism that must obtain all of its energy from carbon reduced by other organisms or in the biomass or other organisms.

heterotrophic plate count (HPC) A method of enumerating heterotrophic organisms by plating a known volume of serially diluted inoculum on an

appropriate nutrient agar medium and counting the colonies to estimate the numbers of colony forming units in the parent sample.

hydration The non-reactive incorporation of water molecules into the structure of a substance.

hypha (*pl.* hyphae) A single fungal filament.

immobilization Incorporation of a substance into living or non-living organic matter or into the structure of inorganic matter or onto a surface, making that substance unavailable for further chemical or physical reaction.

immunofluorescent microscopy, direct Detection of organisms based on the staining of the organisms by means of a dye bound to an antibody specific to those organisms.

inoculation loop A thin metal wire loop at the end of a handle used to transfer a small amount of viable microbial material to a new medium for culture or isolation or to a microscope slide for observation.

inoculum A material containing viable microbial propagules used to create growth of the microbe(s) of interest in a new environment.

intercalate When a molecule can exist in the space between adjacent base pairs in the DNA double helix, causing an eventual insertion or deletion of a base pair. The reading frame of the DNA is shifted by one base pair. Some mutagens and DNA stains, such as acridine orange and ethidium bromide, bind to DNA in this fashion.

isolate A pure, single-species/strain colony of a microorganism.

lactophenol mounting fluid A medium for mounting and staining fungi for microscopic examination.

lag phase of growth A finite period of time where no increase in cell numbers is observed in culture, often due to low initial cell densities.

Levine's eosin methylene blue (EMB) agar A medium used in the confirmed test of the coliform MPN test.

macronutrient A nutrient required by an organism in relatively large quantities, such as nitrogen.

master mix A mixture of ingredients used in PCR utilized for multiple PCR reactions.

mean The arithmetic average of a data set.

mean generation time (doubling time): The amount of time for microbial cell division to occur.

mEndo broth-MF Culture broth which stains coliform colonies red or pink with a gold-metallic sheen.

mFC agar Agar which stains fecal coliform colonies growing on it blue.

microfuge A miniature centrifuge used in the centrifugation of small quantities of sample.

micronutrient A nutrient needed by an organism in small quantifies. Examples include vitamins and trace metals.

minimal salts medium A culture medium containing only mineral nutrients and no oxidizable carbon sources.

moisture content *In soil*: The water content.

mordant A substance that enhances the binding and hence stability of a dye to the dyed material.

myceolium A collective noun referring to all of the hyphae in a fungus.

nitrification The oxidation of reduced forms of nitrogen such as ammonium.

nucleoside *In nucleic acids*: A unit composed of the base + sugar.

nucleotide *In nucleic acids*: A monomeric unit composed of the base + sugar + phosphate.

obligate anaerobe An anaerobic organism that cannot survive in the presence of oxygen.

obligate parasite A parasitic organism that can only survive in parasitizing the host.

oligonucleotide A short, single-stranded segment of DNA or RNA composed of a small number of nucleotides.

oocyst A cyst formed after gamete union in some protozoa, such as *Cryptosporidium*.

oxidation The removal of valence electrons from an element.

oxidizable organic matter Organic matter capable of being oxidized by microorganisms to release energy for metabolism.

ozonation *In water treatment:* Treating water with ozone (O_3) as a means of disinfection, thereby avoiding the formation of potentially carcinogenic chlorinated by-products formed from native organic matter in the water through many chlorination processes.

peptone-yeast agar (PY) A general-purpose medium for the culture of a wide variety of heterotrophic organisms.

Petri plate A glass or plastic culture dish composed of two halves, a bottom and a larger diameter top, whereby the top overlaps the bottom creating an environment that can be used to incubate aerobic organisms yet maintain sterility.

pH A negative base-ten logarithmic expression of the H^+ concentration of a solution.

phenolic rose bengal stain A magenta stain used in general purpose staining of microorganisms for microscopic observation.

photoautotrophic An organism that uses light as an energy source and carbon dioxide as a carbon source. These include some bacteria, algae, and higher plants.

physiological saline A NaCl solution (0.85% w/v) corresponding to the osmotic potential of blood. This is sometimes simply called saline.

physiological test A microbiological assay which measures some aspect of microbial metabolism.

plaque forming unit (PFU) method A method used in the quantification of enteroviruses where a cell monolayer is treated with a known volume of sample possibly containing viable enteroviruses. The cell monolayer is covered with agar and stained with a dye that stains only living cells. The clear zones or plaques that form where cells have been killed are then counted.

polymerase chain reaction (PCR) A process for amplifying small amounts of DNA or RNA to measurable, identifiable quantities through the repeated cycling of three steps: denaturation, annealing, and extension.

ppm Parts per million. An expression of concentration that is slowly being phased out in favor of more definite terms. *In solutions*: ppm is generally taken to mean µg solute L^{-1} solution. *In solids*: ppm is generally taken to mean mg analyte kg^{-1} total mass.

precipitate A solid substance formed in solution through chemical reaction.

presence–absence (P–A) test A water quality test that is concerned with the presence or absence of a pathogen in a given volume of water.

presumptive test The first test in the MPN test for coliforms in water. It screens water samples for the possible presence of coliforms before the confirmed test is performed by analyzing for gas-producing lactose fermenting bacteria.

primer *In PCR*: An oligonucleotide annealed to a template strand of single stranded DNA or to RNA and used to initiate extension.

prokaryote An organism lacking cell organelles and a defined nucleus. Bacteria and actinomycetes belong to this class.

phototrophic Organisms that can synthesize all needed biochemicals, such as amino acids, nucleotides, vitamins, and cofactors given a metabolizable carbon source.

recalcitrant *In microbiology*: resistant to degradation and metabolism by microorganisms.

reduction Addition of electrons to the valence shell of an element.

rod *In bacteria*: A bacterium that is proportionately longer than it is wide.

rose-bengal-streptomycin agar A medium used for culturing and enumerating fungi.

saline See *physiological saline*.

sand The coarsest of the three textural size fractions of soil: soil particles that are between 2 and 0.05 mm in diameter (USDA system).

sand filtration, rapid *In water treatment*: The use of sand filters to remove fine particulate matter from water that are too fine to settle and be removed during sedimentation.

sedimentation *In water treatment*: Settling of floc prior to sand filtration to remove large particulate matter-containing flocs from the water prior to rapid sand filtration. See also *flocculation*.

silt The second coarsest of the three textural size fractions of soil: soil particles that are between 0.05 mm and 0.002 mm in diameter (USDA system).

soil aggregation Binding of single elementary soil particles (sand, silt, clay) into aggregates through microbial cements and fibers, mineral deposits, and electrostatic attraction.

soil texture The proportion of sand, silt, and clay in a soil on a mass basis.

sporozoite The product of cell division and growth from an oocyst in some protozoa.

SSLB **S**ingle **S**trength **L**actose **B**roth. Medium used in the presumptive test of the coliform MPN test.

stain An organic dye used to aid in the visualization of microscopic structures or cells by differentially coloring different components, thereby adding contrast as most biological substances are colorless.

standard solution A solution of known identity and concentration.

stationary phase of growth A finite period of time where in, culture, the number of microbial cells being produced equals the number of cells that become nonviable i.e., no net growth.

swab technique Use of a specially moistened swab to sample surfaces for bacterial contamination.

***Taq* polymerase** A DNA polymerase isolated from *Thermus aquaticus*, a bacterium native to deep-sea hot vents, that is used in PCR due to its innate heat stability.

terminal electron acceptor The substance which accepts the electrons released by the oxidation of an energy source, such as glucose, at the end of

the respiratory chain. In aerobic organisms, oxygen serves this purpose, being reduced to water. In anaerobic organisms, other compounds, such as nitrate or sulfate, may serve as the terminal electron acceptor.

thermal cycler A programmable device for controlling reaction conditions in PCR.

trophozoite A motile, feeding form of a protozoan organism.

USDA **U**nited **S**tates **D**epartment of **A**griculture.

USEPA **U**nited **S**tates **E**nvironmental **P**rotection **A**gency. Also called EPA.

viable but nonculturable Microorganisms that are viable but cannot be cultured, often due to the effects of environmental stress.